Observations of Novel Magnetofection Agents, and Implications for Magnetofection Agent Design and Assessment

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1 Table of Contents

1 Table of Contents..............................................................................................................3

2 Acknowledgements ........................................................................................................7

3 Nomenclature..................................................................................................................8

3.1 Abbreviations................................................................................................................8

3.2 Terms.............................................................................................................................8

4 Abstract..........................................................................................................................9

5 Introduction & Literature Review...................................................................................11

5.1 Gene Transfer................................................................................................................12

5.1.1 Application of Gene Transfer....................................................................................12

5.1.1.1 Clinical Relevance for Gene Transfer.................................................................12

5.1.1.2 Transfection Efficiency.......................................................................................14

5.1.1.3 Methods of Gene Transfer...................................................................................15

5.1.2 Non-Viral Transfection Platforms............................................................................17

5.1.2.1 Traits of Non-Viral Platforms...........................................................................18

5.1.2.2 Current Non-Viral Transfection Platforms.........................................................18

5.1.2.3 The Performance of Non-Viral Transfection Agents.........................................20

5.1.3 Targeted Transfection..............................................................................................21

5.1.3.1 The Relevance and Performance of Passive Targeting......................................21

5.1.3.2 Active Targeting by Moieties............................................................................22

5.1.3.3 Active Targeting by External Force.................................................................22

5.2 Magnetic Nanoparticles..............................................................................................24

5.2.1 Analysis of Magnetic Properties Relevant to Nanoparticles..................................24

5.2.1.1 Important Magnetic Values..............................................................................25

5.2.1.2 Superparamagnetism.......................................................................................25

5.2.1.3 Magnetometry by SQUID................................................................................27

5.2.2 Materials for Magnetic Nanoparticles.....................................................................28

5.2.2.1 Synthesis and Storage of Superparamagnetic Nanoparticles.............................29

5.2.2.2 Biocompatibility of Magnetic Nanoparticles....................................................30

5.2.2.3 Synthetic Routes for SPIONs............................................................................31

5.2.2.4 Common Surface Coatings for SPIONs..........................................................32

5.3 Magnetofection...........................................................................................................33

5.3.1 Previous Effectiveness of Magnetofection...............................................................33

5.3.1.1 Improved Transfection Efficiency.................................................................34
6.3.1.2 Static and Oscillating Modes

6.3.1.3 Targeting with Magnetic Force In Vivo

5.3.2 Magnetofection Agent Construction

5.3.2.1 Synthesis In Situ

5.3.2.2 Covalent Linkers

5.3.2.3 Electrostatic Association

5.3.3 Establishing a Mechanism for Magnetofection

5.3.3.1 Vector Sedimentation

5.3.3.2 Endocytotic Passage

5.3.3.3 Mechano-sensitivity in Cellular Surfaces

5.3.3.4 The Fate of Magnetic Materials Within Cells

5.3.4 Previous Optimal Conditions for Oscillating Magnetofection

5.3.4.1 The Role of Frequency and Rotational Axes

5.3.4.2 The Role of Field Amplitude and Strength

5.4 Summary

6 Materials and Methods

6.1 Superparamagnetic Nanoparticles

6.1.1 Standard SPION Synthesis for this Project

6.1.1.1 Initial Synthesis

6.1.1.2 Coating

6.1.2 Alternative Metal Nanoparticle Synthesis

6.1.2.1 Cobalt Ferrite Nanoparticle Synthesis

6.1.3 Quality Assurance of SPION

6.1.3.1 SQUID Magnetometry

6.2 Synthesis of Novel Transfection Agents

6.2.1 PEI-PGMA Nanospheres

6.2.2 Dendronised Polymer

6.3 Cell Culture

6.3.1 Care and Passage of Cells

6.4 Transfection Protocols

6.4.1 General Transfection Protocol

6.4.2 Cell Survival Assay

6.4.3 DIf Staining

6.4.4 Statistics

6.5 Analytic Devices

6.5.1.1 Superparamagnetic Quantum Interference Device (SQUID)

6.5.1.2 Electron Microscopy

6.5.1.3 Dynamic Light Scattering
7 Results & Discussion .................................................................................................................. 62

7.1 SPION .................................................................................................................................. 63
  7.1.1 SQUID ............................................................................................................................... 65
  7.1.1.1 Olate SPION .................................................................................................................. 65
  7.1.1.2 Citrate SPION ............................................................................................................... 66
  7.1.1.3 SPION Magnetic Values ............................................................................................... 67
  7.1.2 DLS and Zeta ................................................................................................................... 68

7.2 PEI-PGMA Nanospheres ........................................................................................................ 69
  7.2.1 DLS and Zeta ................................................................................................................... 69
  7.2.2 Epifluorescence Microscopy ........................................................................................... 72
  7.2.3 Discussion of PEI-PGMA Nanospheres ......................................................................... 73

7.3 Novel Dendronised Co-Polymers / C-SPION ....................................................................... 74
  7.3.1 DLS and Zeta ................................................................................................................... 75
  7.3.2 Epifluorescence Microscopy ........................................................................................... 76
  7.3.3 Flow Cytometry .............................................................................................................. 77
  7.3.4 Discussion of Dendronised Polymer ............................................................................... 78

7.4 Lipofectamine 2000 / nTMag+ .............................................................................................. 79
  7.4.1 DLS and Zeta ................................................................................................................... 81
  7.4.2 Epifluorescence Microscopy ........................................................................................... 81
  7.4.2.1 Verification of Effective Dil Staining of Lipofectamine 2000 ........................................ 82
  7.4.2.2 Verification of Liposome distribution during Magnetofection ..................................... 85
  7.4.2.3 Verification of Co-Transfection Efficacy of MCF7 Cells ............................................ 87
  7.4.3 Microplate Photometry .................................................................................................... 88
  7.4.3.1 Verification of Effective Dil Staining of Lipofectamine 2000 ........................................ 88
  7.4.3.2 Cytotoxicity .................................................................................................................. 89
  7.4.3.3 Effective Use of nTMag Plus ......................................................................................... 90
  7.4.4 Flow Cytometry ............................................................................................................. 92
  7.4.4.1 Verification of Effective Dil Staining of Lipofectamine 2000 ........................................ 92
  7.4.4.2 Magnetofection .......................................................................................................... 93
  7.4.4.3 Field Optimisation ....................................................................................................... 95
  7.4.4.4 Co-Transfection ......................................................................................................... 97
  7.4.5 Discussion of nTMag Plus .............................................................................................. 98
  7.4.5.1 Insights into the mechanism of magnetofection .......................................................... 98
  7.4.5.2 Acceleration of Gene Delivery by nTMag+ ................................................................. 100

7.5 Future Work .......................................................................................................................... 102
  7.5.1 Use of Conjugated SPION Particles as Magnetic Moieties ........................................... 102
7.5.2 Magnetofection Conditions with Superior Resolution .......................................................... 102
7.5.3 Cobalt Ferrite Nanoparticles ............................................................................................... 103
7.5.4 The Effect of Oscillating Magnetic Fields and SPION on Endocytotic Pathways .................. 106

7.6 Conclusions ............................................................................................................................. 107

8 References ..................................................................................................................................... 108
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3 Nomenclature

3.1 Abbreviations

CI: Confidence Interval
DiI: 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
DMSA: Dimercaptosuccinic acid
DOSPA/DOPE: N-[1-(2,3-dioleoyloxy)propyl]-N',N',N'-trimethylammonium chloride
EGFP: Enhanced Green Fluorescent Protein
GMA: Glycidylmethacrylate
HEMA: Hydroxymethacrylate
nTMag: A proprietary polymer coated iron oxide nanoparticle for Magnetofection
nTMag Plus: An nTMag variant for augmenting extant gene delivery agents.
PAA: Polyacrylic Acid
PAMAM: Polyamidoamide
PDI: Polydispersity Index
PEG: Polyethylene Glycol
PEI: Polyethyleneimine
PGMA: Polyglycidylmethacrylate
RhB: RhodamineB
SPION: Superparamagnetic Iron Oxide Nanoparticles
w/v: Percent Weight/Volume.

3.2 Terms

DNAse (plural: DNAses): A class of DNA degrading enzymes.
Indication: In medicine, a condition which would make the use of a particular treatment or procedure advisable.
MilliQ Water: Type 1 ultrapure water produced by a Milli-Q water filtration station.
Moiety: A functional group or component.
4 Abstract

Gene delivery offers solutions for a variety of indications, but primarily chronic disease and cancer. While early gene delivery approaches focused on use of viral capsids as vehicles, non-viral transfection agents are desirable for the minimisation of immune and regulatory complications. Magnetofection approaches, where the efficacy and efficiency of gene delivery are enhanced by the interactions of the transfection agent with an external magnetic field, are currently unexplored clinically, but leverage technologies also in use in hyperthermia and targeted drug delivery for similar indications, which are in clinical trials.

The identification of suitable use cases and formulations for magnetofection treatment is therefore still of relevance, and is the subject of this thesis. Here, several candidate gene delivery systems with magnetic moieties were assessed with respect to their effectiveness in vitro.

A PEI coated PGMA nanosphere with RhB as a fluorophore, containing SPIONs, with previously published efficacy in achieving cellular entry was assessed as a carrier for DNA into cells. This vehicle was able to achieve uptake effectively (~95% of cells) even in the absence of magnetic fields, but was seen to achieve poor expression (~2% of cells). Modifications of the PEI coat, particularly fluorination, may be able to enhance endosomal escape capabilities of this type of vehicle in future, but presently it is uncompetitive.

A novel HEMA-GMA co-polymer decorated with PAMAM dendrimers from a family of vehicles with previously reported effectiveness in achieving DNA delivery and expression, was associated electrostatically with citric acid coated SPIONs, to create a magnetofection vehicle. Ultimately, the addition of SPION to the transfection polyplex steadily decreased the efficiency of transfection. The use of magnetic field conditions conforming to best practices observed with other magnetofection agents in conjunction with these particles further reduced their effectiveness in achieving expression of delivered plasmids. In light of these observations, further development in the direction of magnetofection agents of this design was halted.

Lastly, Lipofectamine 2000, a well studied, industry standard among non-viral transfection agents, was paired with a commercial magnetic moiety developed for magnetofection use, nTMag Plus to create a magnetically aided gene delivery system. The SPION formulation, nTMag Plus, is based on an independent magnetofection agent that has achieved efficacy in prior studies, nTMag, which is a polymer coated SPION. However, the use of nTMag Plus
to enhance the effectiveness of existing transfection agents is presently not well reported. Lipofectamine 2000 was treated with a lipophilic dye ‘DiI’, which was able to elucidate the fate of the magnetofection vehicles.

The Lipofectamine 2000 and nTMag Plus gene delivery vehicle was able to achieve a rate of expression from gene delivery significantly (p < 0.01) in excess of Lipofectamine 2000 alone through use of an oscillating magnetic field, for both EGFP and mCherry plasmids. This improvement was conditional to short transfection times, with magnetofection protocols taking 1 hour or less achieving a 2-fold increase in transfection efficiency relative to non-magnetic protocols conducted over the same time period.

It was noted that the level of lipoplex uptake by cells was modified by changing conditions in magnetofection in a manner not necessarily proportional to corresponding increases or decreases in expression of delivered plasmids. Observations of stained liposomes incorporated into cells during magnetofection do not appear to support the theory that stimulation of SPION by oscillating magnetic fields affects the rate of endocytosis by cells, as absorption was not significantly different between static and oscillating magnetic field modes. While observations are consistent with the primary factor enhancing transfection during magnetofection being sedimentation of vectors, other factors may still be relevant, as the ratio of vehicle uptake rate to expression rate was not the same across magnetic field conditions.

In short, nTMag Plus was found to be able to improve the gene delivery performance of Lipofectamine 2000 2-fold for transfection times 1 hour or less. PEI-coated PGMA nanospheres as used here were ineffective as gene delivery agents. The addition of SPION to a dendronized copolymer examined was only detrimental to its transfection performance rather than offering a promising magnetofection agent. Finally, the relationship between lipoplex uptake rates and gene expression rates in magnetofection was observed to vary with magnetic field conditions.
5 Introduction & Literature Review

Gene therapy is a technique with clinical relevance, centred around delivery of therapeutic DNA or RNA via a process termed transfection. It is an important emerging treatment for several chronic, single-gene diseases, such as cystic fibrosis, but also for cancer, a leading cause of death in the developed world. Gene therapy as a cancer treatment can manipulate mutated genes to address tumours, specific tissue masses dominated by cells with abnormal growth patterns. Many extant cancer treatments have severe systemic side effects due to the whole-body activities of agents intended only for tumour treatment.

Magnetofection is a technique which utilises external magnetic forces to direct and amplify the activity of reagents for gene therapy. Therefore, magnetofection offers a strong potential for spatial targeting. As spatial targeting is an ideal component of an anti-tumour treatment, development of magnetofection has importance in producing novel and relevant therapeutics.

The following are the principle aims addressed by this thesis.

- The comparison of the performance of magnetic and non-magnetic transfection protocols incorporating novel non-viral transfection agents.

- Examination of how the commercial SPION formulation ‘nTMag Plus’ affects the process of transfection.

Within this project, several gene delivery platforms not previously assessed as magnetofection agents were examined for effectiveness in vitro. A high performing agent, nTMag Plus, was observed in greater detail to identify underlying factors driving effective magnetofection.

In order to establish this study’s aim of evaluating protocols for non-viral, magnetically aided gene transfection, I will cover literature identified as relevant to this topic. First, the state of past and current gene transfer fields is explored. Following this, the subfield of magnetofection specifically is examined in greater detail. Finally, the properties of magnetic nanoparticles suitable for enacting magnetofection are covered.
5.1 Gene Transfer

The transport of nucleic acid into cells is necessary for many cutting edge medical technologies that particularly include gene therapy and tissue graft production. Gene transfer’s inception in studies of Pneumococcus strains showed a transfer of virulence factors between strains, where this process was referred to as ‘transformation’. Studies demonstrating the mediation of gene transfer between live Salmonella bacteria through a glass filter by bacteriophages led to ongoing interest in viruses as gene transfer agents. The ‘transformation’ of cells through ‘infection’ with viral vectors forms the term ‘transfection’ which is used to refer to gene delivery today, even when utilising less common non-viral vectors.

Interest in transfection intensified following the first demonstrated correction of a human genetic defect in cells by the addition of DNA from an external source, establishing a relevance for transfection in medicine. Modern developments in eukaryotic gene transfer focus on therapeutic use, and so the effectiveness of gene transfer protocols is often assessed in terms of factors important in such a setting, namely low cytotoxicity, and high rates of uptake and expression.

5.1.1 Application of Gene Transfer

The first institutionally approved clinical protocol with human subjects was with the use of cultured tumour infiltrating lymphocytes transfected virally and re-seeded into advanced melanoma as a delivery vehicle for tumor necrosis factor. Since then, gene therapy clinical trials have become substantially more accepted, with many clinical indications and vehicles in use presently. However, the use of cells transformed ex vivo, and cancer as a target for therapeutic treatment, remain a common theme throughout much of modern gene transfer.

5.1.1.1 Clinical Relevance for Gene Transfer

Clinical targets for gene transfer are varied in scope and as referred to above include the use of externally cultured cells as tailored delivery agents for therapeutic compounds, and the use of cultured cells as a replacement for cells otherwise present in the patient that are genetically deficient. A family of trials where transformed T-cells were used to treat adenosine deaminase (ADA) deficient severe combined immunodeficiency (SCID) were the first of this type. This study demonstrated a common pitfall, where low expression
ultimately meant that the gene therapy component of the treatment was inadequate as treatment longer term, even though the transformed cells were retained for a significant time, exhibiting a half-life of up to 12 months \(^8\).

Over time, an improved understanding of conditioning regimes for recipients of modified cells has led to more recent clinical success in the treatment of chronic diseases such as ADA-SCID \(^9\), and β-thalassemia \(^10\), with effective treatment of the conditions coming at the cost of aberrant growth factor activations \(^11\). **However, most gene therapy targets in the present day are malignant cancers and tumours.**

Cancers have over the last decade taken up a steadily larger proportion of gene therapy trials, as can be seen in Figure 1. They offer an attractive clinical target because the traditional treatments for cancer, irradiation and chemotherapy, have severe off-target effects on patient health. Cancer remains a relevant clinical target because it is the most significant cause of death in the first world \(^1\).

A looming opportunity and threat for gene therapy, that of germ-line cell gene editing to produce inheritable changes in the human genome, remains banned in nearly all nations with significant scientific regulatory bodies \(^12\). While consensus among the international community is to ensure that germ-line gene editing is not inherited \(^13\), China, a rapidly growing producer of genetic technologies, has instituted guidelines which are considered difficult to enforce \(^12\).
Another expanding sector of gene therapy currently gaining attention is the prospect of DNA vaccines, where gene therapy is used to deliver modified viral genomes into mammalian cells in order to trigger production of viral proteins throughout the body over an extended period \(^{14}\). The most notable such trial could be the Genetic Immunity sponsored trial for the delivery of LC002 (a plasmid encoding inert HIV capsid proteins) by polyethyleneimine (PEI), which serves as a DNA vaccine, slated for Phase III trials after success in earlier trials \(^{15}\). DNA vaccines can also benefit greatly from co-transfection, a challenging technique where multiple plasmids are delivered simultaneously within the same vehicle \(^{16}\). DNA vaccines drive trials of non-viral gene transfer agents like those in use for this project \(^{17}\).

Currently, chronic and monogenic diseases pose interesting targets for gene transfer applications. However, the majority of interest, and therefore the indication around which research and development is oriented, is cancer treatment, generally as an adjuvant to assist extant chemotherapy, surgery or radiotherapy methods \(^{18}\).

5.1.1.2 Transfection Efficiency

Transfection efficiency is the measure of a gene delivery agent’s effectiveness, for studies \emph{in vitro} it is assessed and reported in literature by two primary means. The overall quantity of transcript expressed by the target cells, and the percentage of target cells with elevated expression of transcript. The latter can be a more useful measure of effectiveness, but the former is more easily quantified and appears more often. It can be challenging to obtain proper quantitative comparison, as the effectiveness of agents in different cell lines also differs.

However, even for studies of similar vectors, plating density and the concentration of plasmid utilised are not necessarily consistent for papers examining transfection. A common assay, luciferase, used to assess overall transcript expression, has less than ideal reproducibility due to both variable luciferase quality and instrument calibrations \(^{19}\). This has made detailed comparisons of separate papers which also report using relative luminescence units (RLU) less valid than they could be.

The presence or absence of serum during transfection is also an important consideration. Serum, a collection of proteins analogous to and often derived from cell free blood, allows for conditions that more closely mimic those found \emph{in vivo}. Transfection agents usually
perform comparatively poorly in serum conditions due to protein agglomeration and other interference. While prospective transfection agents are usually assessed in serum free conditions to maximise the effective resolution of increases or decreases in transfection, serum resistance, where transfection efficiency is not as impeded by the presence of serum is ultimately an important trait of gene delivery vehicles with prospects for use in vivo.

While the aforementioned factors complicate comparison between transfection studies, the available methods of quantification nonetheless offer a means to contrast the effectiveness of gene transfer agents.

5.1.1.3 Methods of Gene Transfer

The earliest gene transfer studies did not utilise vehicles (also referred to as transfection agents, or vectors) to package DNA for transfer into target cells. While prokaryotes are relatively receptive to this free DNA, eukaryotic cells, which are of greatest interest for medical applications, only uptake and express exogenous DNA under stressful conditions and at extremely low rates, as high as 0.0085% for serum conditions. Injections of naked plasmid directly into tissue have seen some limited success in vivo when targeting tissues well removed from the factors responsible for DNA degradation in plasma, such as muscle. Ultimately, more complex viral or non-viral protocols are necessary.

Viral vectors constructed from bacteriophages and eukaryotic viruses were identified early as a means of delivering genes, and continue to be of relevance in the modern day. As the primary vectors for gene transfer in nature, and it has been understood for some time that gene transfer utilising viral capsids has promise as a treatment for genetic defects. They have excellent mechanisms for insertion into target cells and genomes, but in nature this is used for self-replication and propagation first and foremost. Due to their natural source there are issues with their use in medical applications.

Mass production of viruses requires compromises between efficiency and risk of complications. Fears of a hypothetical worst case scenario of unwanted transformation or contagion beyond the patient have led to a greater controversy in use of viral vectors, and incidents of cancer arising from insertional mutagenesis during viral therapies have further increased concerns. More rationally, because the human immune system recognises viruses as threats, use of viral vector based protocols can provoke extreme immune responses in vivo that can impede treatment, or result in lethal complications.
For these reasons, alternative research into non-viral transfection platforms has been of interest since the early days of gene transfer, however their transfection efficiency is usually much lower. The earliest categories of non-viral transfection agents, cationic liposomes and cationic polymers complexes, remain the most relevant in modern gene technologies.

These chemically synthesised non-viral vectors provide a modular and well-understood alternative to viruses.

Generally included under the umbrella of non-viral delivery are protocols for forcing insertion of DNA into cells without the use of viral capsids or synthetic vehicles, notably electroporation using strong electrical currents or sonoporation which uses sound waves to propel DNA through the cell membrane. These methods, also often referred to as non-viral, only make up around 0.4% of clinical trials due to limited applicability in vivo. Their efficiency of transfection ex vivo is also low compared to viral methods. Their competitive advantage is that no foreign material other than therapeutic DNA is necessarily added to cells.

Currently, vehicle based non-viral protocols comprise only a small portion of gene delivery agents used clinically. This is around 5% as per a census by the Journal of Gene Medicine, which is less than either adenoviruses or retroviruses as per Figure 2. Adenoviruses, and retroviruses are the most popular vectors in use for clinical trials, due to a relative ease of use. Overall, most vectors used for clinical tests are viral in nature, with non-viral vectors being comparably rare outside of research and development.
5.1.2 Non-Viral Transfection Platforms

Non-viral transfection agents are a broad and expansive category containing all possible vehicles and physical methods that can improve the uptake of DNA without the use of viral capsids. For the purposes of this thesis, non-viral transfection overwhelmingly refers to the use of vehicles rather than techniques that mechanically deliver naked plasmid DNA. The majority of synthetic vehicles for transfection yet explored can be categorised as a polymer complex, lipid complex, or a combination of the two, but alternatives such as silicon nanowires used to mechanically transfer DNA by impaling cells in vitro and in vivo exist.

To protect DNA from nuclease activity during delivery, it is ideal to fully enclose it. Therefore, most agents possess a cationic charge that attracts and coils DNA around the carrier and vice versa. The result is a self-assembled complex, either a polyplex or lipoplex. These polymer and lipid constructs can incorporate other functional moieties, such as SPIONs, which allow for magnetically driven transfection as in the focus of this project.

Non-viral transfection agents must address specific barriers to successful transfection that would otherwise impede expression of naked plasmid DNA. First, firm contact with target cells must be achieved. Complexes must be capable of being internalised from the cell surface, which typically proceeds by endocytosis mediated by the cell itself. Because of this entry by endocytosis, transfection agents are then located within endosomes, which are a hostile and contained environment for DNA, which necessitates endosomal escape.

Endosomal escape is an open ended process but is usually achieved by the ‘proton sponge’ effect, where agents buffer against the acidification of endosomes, leading to endosomes osmotically swelling until they rupture. Finally, DNA must achieve nuclear entry, which is relatively easy in rapidly dividing cells due to the compromise of the nuclear membrane during mitosis, but requires use of the nuclear pore complex otherwise. DNA must be free of its delivery vehicle by the time of nuclear entry to achieve efficient nuclear entry and avoid impeding expression.

An agent which overcomes all these barriers will successfully deliver DNA into cells. However, the rate at which each stage is successful, as well as control of other traits such as the relative cytotoxicity caused in the process, decides the ultimate quality of a transfection agent.
5.1.2.1 Traits of Non-Viral Platforms

Non-viral platforms possess relatively high cytotoxicity and low transfection efficiency compared to viral reagents. Their cytotoxicity is unsurprising as they are often highly charged, DNA associating compounds, and thus disruptive to normal cellular function. Non-viral platforms are unlikely to trigger abnormal immune activity unless specifically designed to do so.

Non-viral transfection reagents tend to package DNA quickly, which is necessary to protect against DNAses present during transport. Because their chemical structure is very simple compared to a viral capsid, it is also comparably easy to add new moieties to non-viral transfection agents for the purposes of targeting, passage through barriers in vivo, and monitoring, making them very modular. Additionally, non-viral vectors generally have a greater carrying capacity for therapeutic DNA transcripts.

5.1.2.2 Current Non-Viral Transfection Platforms

Among non-viral gene therapies, transfection using lipoplexes is the most clinically trialled (See: Figure 2), and possess the greatest achievable transfection efficiency in cell models. Of these, the Lipofectamine series of anionic lipoplex formulations based on DOSPA/DOPE (Lipofectamine, Lipofectamine Plus, Lipofectamine 2000, Lipofectamine 3000) are widely used as transfection agent formulations sold commercially for laboratory use. 1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine conjugated PEG is a popular cationic lipid reagent for clinical use, such as in treatment of cystic fibrosis via the inhalation of a nebulised dose.

Among polymeric transfection agents, PEI based nanoparticles are a common basis for gene therapy platform research, ex vivo and in vivo transfection, often conjugated to other agents or moieties to enhance function. PEI is a high performing transfection agent offering good protection from DNAse activity and endosomal escape capabilities. While it is rendered less ideal by its high cytotoxicity, it is very relevant in high interest clinical cancer targets such as lung and pancreatic cancer. While conjugates containing other linear polymers such as dextran and poly-L-lysine see use in research and ex vivo delivery, they are generally uncompetitive with PEI save for small plasmid delivery.

Branching of polymers is a method of varying the structure of polymeric transfection agents, and dendronised polymers can condense DNA very effectively, allowing transit of
larger DNA cargo intact into cells. Dendrimers and dendronised cationic polymers are also believed to possess attractive properties as transfection agents, with highly effective encapsulation and protection of DNA coupled with exposure of moieties necessary for endosomal escape. PAMAM is an example of a dendrimer commonly used for transfection, usually conjugated to a targeting moiety.

Figure 3: Polymer design and transfection efficiency in MCF-7 cells for a dendronized polymer used in this thesis. (a) Synthesis and architecture: polymers are random statistical copolymers of HEMA and GMA where $x = \frac{1}{4}$ mol% GMA. Poly(amide amine) dendrons of different generation are clicked onto the linear polymer backbone. Reaction conditions: (i) NaN₃, NH₄Cl, DMF, 60 °C, 72 h; (ii) PMDETA, CuBr, DMF, r.t., 72 h; (iii) ethylenediamine, MeOH, 0 °C. (b and c) Representations of differing dendronized polymers. Schematics demonstrate how flexibility can be systematically altered by varying dendron generation and dendron density. (d) Transfection efficiency of polymers delivering EGFP-encoding plasmid to MCF-7 cells. From Kretzmann, J. A. et al. Synthetically controlling dendrimer flexibility improves delivery of large plasmid DNA. Chem. Sci. 8, 2923–2930 (2017), Figure 1, reproduced by permission of The Royal Society of Chemistry.

Combinations of the polymeric and lipid reagent categories have also been explored in vitro, using combinations of oppositely charged polymer and liposome to augment DNA delivery. This assembly can also minimise the cytotoxicity of the compounds, which in single transfection agents has been observed to be proportional to the cationic charge of the agent.

A recent vector design consisting of PEI chains surrounding a core nanosphere of poly-glycidyl methacrylate (PGMA) which contains both magnetite nanoparticles and Rhodamine B for magnetic and fluorescence detection respectively has been analysed. The nanosphere vector proved easy to image in situ with a variety of techniques due to its size, high contrast features, and fluorescence, while also displaying effective transfection activity in a static magnetic field. Recently it has also been shown that grafting PEI with fluoroalkyl chains, which are extremely lipophilic, can enhance transfection activity.
leads to an expectation that polymer nanospheres with the additional modification of fluoroalkyl chain grafting would be suitable as a high performing transfection vector prior to optimisation of magnetic field conditions. This is one of the possibilities that this thesis investigates.

![Figure 4: A scheme for fluorescent PGMA nanospheres containing iron oxide nanoparticles, that were utilised in this thesis. Fluorescent, superparamagnetic nanospheres were prepared by an emulsion route and made use of the reactive epoxide groups of PGMA to anchor PEI. Adapted with permission from (Evans, C. W. et al. Multimodal analysis of PEI-mediated endocytosis of nanoparticles in neural cells. ACS Nano 5, 8640–8648 (2011)). Copyright 2011 American Chemical Society.]

### 5.1.2.3 The Performance of Non-Viral Transfection Agents

With respect to transfection efficiency, optimal conditions for non-viral gene delivery agents, generally involve easy to transfect cells in serum free conditions, coupled with high performing transfection agents such as lipoplexes and fluorinated dendrimers. In these conditions, cells can be transfected over 90%60, and achieve expression levels of more than $1 \times 10^8$ RLU per mg of protein $^{61}$ when transfected in Luciferase assays. However, similar compounds can and often are $^{47}$ reported to produce less than a third of these values under less than ideal transfection circumstances such as shorter timeframes $^{62}$, differing cargo $^{60}$ and difficult to transfect cell types $^{63}$.

For comparison, adeno-associated viruses, when used in luciferase assays, are often able to achieve 4 times this level of total expression in similar cell lines $^{35}$. While RLU values This could indicate a need to improve transfection efficiency for non-viral platforms.

Performance in vivo is an important gauge of the effectiveness of a gene transfer agent. As an adjuvant to chemotherapy, there is anecdotal success in sending tumours into remission with non-viral gene delivery $^{64}$. In animal models, non-viral transfection has limited utility as a lone and permanent solution to malignant tumours, with only prolonged survival achieved, and generally with less effectiveness than equivalent viral models $^{65,66}$. Many are impressive with respect to the observed expression at the target site however $^{65-67}$, which
may imply that expression of the plasmids currently being utilised in gene therapy is inadequate as a means of totally suppressing tumours.

However, in the treatment of hereditary conditions, or genetically linked diseases, non-viral vectors have proven to have efficacy. Animal models and clinical trials addressing cystic fibrosis, arthritis, and mitochondrial defects have shown significant improvements in condition through the action of non-viral vectors. There is also a great potential for the expansion of gene therapy trials into conditions currently proven addressable by gene therapies leveraging viral vectors, such as Parkinson’s disease, Alpha-1 antitrypsin deficiency, and haemophilia.

5.1.3 Targeted Transfection

One way to enhance non-viral vector performance in transfection, particularly in vivo, is to localise their effect to the target area, such as a tumour, more effectively. Viruses accept new moieties with targeting capabilities less readily, and so can be outperformed in this regard by non-viral transfection agents. Magnetofection, as in this project, is an example of a method targeted by external magnetic force, which offers spatially resolved targeting capacity by way of implanted magnets, or carefully shaped magnetic flux from a sufficiently powerful external magnet.

5.1.3.1 The Relevance and Performance of Passive Targeting

Before specific efforts are made to modify agents to selectively increase transfection efficiency of target tissues, many target and agent combinations already benefit from a passive form of targeting. In the case of tumours, the enhanced permeability and retention (EPR) effect of tumours easily results in disproportionate accumulation of nanoparticles and transfection activity for tumour cells relative to whole body tissue in vivo.

Certain organs, such as the liver or kidneys, accumulate transfection agents at a rate controlled by basic features of the agent design, such as charge, which makes targeting these regions simple as they are naturally responsible for blood clearance of many nanoparticles. The blood brain barrier meanwhile, is a considerably effective filter against bloodborne agents, and so by default most transfection agents will have less activity and by extension less toxicity in the brain. Avoiding filtration by the liver and kidneys through the use of neutrally charged ‘stealth’ complexes such as PEGylated polymers
extends the time taken for clearance of nanoparticles, but in extending the circulation time in blood, this can improve transfection of highly vascularised areas such as the lungs and digestive tract 79,80.

In whole animal or clinical gene therapy, the delivery site for the vehicle bears consideration: considerably greater transfection activity can be expected within close proximity to injection sites, as exposure precedes clearance by the liver or kidneys 66. For this reason, muscle and fat deposits that are not close to vasculature, skin, lungs, as well as the eye and some internal mucous membranes, can be preferentially transfected by proper injection site selection alone 21,38. This is the most rudimentary form of spatial targeting available for gene delivery protocols besides reliance on EPR, but requires no vehicle modification. By making specific steps to modify vectors for their target area, more specificity can be achieved.

5.1.3.2 Active Targeting by Moieties

Where passive targeting is inadequate, it is possible to modify transfection agents with moieties that preferentially accumulated them near certain cells or organs. For example, linking with transferrin, an iron transport glycoprotein, is a very popular means of adding active targeting to transfection agents 78,81,82. Receptors for transferrin exist in most cells, but are upregulated in many rapidly dividing tumours 81. Transferrin can also be used to accumulate material onto the blood brain barrier, where there is also a high level of transferrin reception 78. Tet1, a neuronal cell binding peptide, has been demonstrated to allow for increased uptake and specificity when conjugated to PEI based transfection agents 83. Simpler targeting factors can also be used, such as pH sensitive peptides 84.

5.1.3.3 Active Targeting by External Force

Installing moieties that chemically target certain tissues and cell types has limitations. In the event of applications targeting a specific tumour, or injury, the spatial location of transfection can be more desirable than a vehicle targeting factors expressed at the target. In these cases, it is appropriate to attach and make use of moieties that can modify activity of the agent based on intervention from outside by a macro-scale force. In this regard, conjugation to metal nanoparticles is common 21.

These nanoparticles can act as receivers for directed radiation 85, soundwaves 86, or magnetic fields 21 from emitters mounted outside the body, and utilise these energies at
their own operational scale, within a limited area targeted by the energy. For instance, they can be used to speed membrane entry through application of force 87, or stimulate DNA uptake through stress by serving as nanoscale heating elements 88. Of interest for this project is the practice of magnetofection, where external magnetic force is applied to a magnetic element of the transfection agent. This process either increases accumulation of agent in areas targeted by magnetic flux, increases activity of the agent when exposed to that flux, or both.
5.2 Magnetic Nanoparticles

Magnetic nanoparticles are used for many different purposes within biotechnology\(^8^9\). In addition to directing sedimentation\(^2^1\) and use as labels for monitoring\(^9^0\), particles can also be agitated to trigger inductive heating through hysteresis\(^8^9,9^1,9^2\). Hyperthermia treatments are more clinically relevant than gene therapy presently\(^9^3\). However, schemes exist where inductively heated particles driving release from structures built around the nanoparticles for targeted payload release\(^9^4\) would allow hyperthermia particle designs with prior testing and approval to be leveraged in future magnetofection efforts.

In general, the ability to manipulate nano-scale objects with macro-scale magnetic arrays, or as magnetic objects with an extremely high surface area to volume ratio presents unprecedented opportunities. Magnetic nanoparticles are also capable of superparamagnetic behaviours, which are distinctly useful in magnetics applications \textit{in vivo}.

5.2.1 Analysis of Magnetic Properties Relevant to Nanoparticles

Several types of magnetic behaviour in result from the spin arrangement of fermions (subatomic particles with half integer spin quantum numbers), such as electrons, neutrons and protons, in atomic structures. Where electrons are paired, there is no NET spin in atoms, and they only exhibit diamagnetism due to slight uncooperative behaviours. Diamagnetic responses are ubiquitous, but weak enough to be insignificant in materials expressing other magnetic behaviours.

Asymmetry from unpaired electrons elicits paramagnetism, where individual magnetic moments exist and can be aligned with external magnetic fields, resulting in increasing magnetisation with applied field. In ferromagnetic materials, the exchange of magnetic forces between these individual moments is strong enough to create magnetic alignment between spins spontaneously, maintaining magnetisation in the absence of external magnetic field.

While magnetite technically is known to behave ferrimagnetically, where material self-organises into a system not fully aligned with field, its distinctions from ferromagnetic behaviour are difficult to discern. Ferromagnetism is thus the default behaviour expected of the magnetic moieties in this project, in the absence of superparamagnetic effects.
5.2.1.1 Important Magnetic Values

Along with the synthesis of novel magnetic materials comes a need to characterise their properties effectively. Values of interest for a magnetic nanoparticle in biotechnology are its Curie temperature, saturation magnetisation, coercivity, and remanence. These values are still often reported in CGS units as a convention of the field of magnetics. For this project, values will be converted to SI units where immediate comparison with values cited in SI is necessary, but otherwise reported in CGS.

Above the Curie temperature, thermal energy overcomes the material's capacity to retain magnetisation. This can be expected to resemble the bulk material's Curie temperature. Curie temperature below physiological temperatures is not ideal as particles will demagnetise in vivo.

The saturation magnetisation is the maximum magnetisation of material by an external field, generally quoted at Standard Temperature and Pressure (STP) as a function of mass or volume although other conditions can become relevant. Increasing applied field brings individual magnetic moments into alignment, but eventually moments have been aligned to the maximum extent and further magnetisation is impossible. This is usually a property of the specific crystal phase of the material. Saturation magnetisation is a good predictor of the eventual motive force that can be applied by magnetic field to particles.

Coercivity is the intensity of an external magnetic field required to neutralise any pre-existing magnetisation from a permanent magnet. Remanence is the magnetisation that can be retained by a material in the absence of applied magnetic fields. Materials with high coercivity and remanence are considered ‘hard’ magnets, while materials with low coercivity and remanence are considered ‘soft’ magnets.

5.2.1.2 Superparamagnetism

Superparamagnetism is a unique consequence of magnetic nanomaterials. Superparamagnetism is observed only when the size and number of domains, in a magnetic material are both sufficiently small, generally occurring in particles between 150 nm and 10 nm in diameter depending on the material. When this is the case, individual particles can be considered to conform to the single domain anisotropy energy equation for a spheroid of:

\[ E_{\text{anisotropy}}(\alpha) = -KV \cos^2 \alpha \]
where $\alpha$ is the angle between the direction of magnetization $\mathbf{M}$ and the easy axis, $V$ is the volume of the particle and $K$ is the uniaxial magnetic anisotropy constant. Consequently, thermal energy below the Curie temperature of the bulk material can flip the orientation of magnetic moments within the material between its two most stable orientations only. These are the anti-parallel anisotropy energy minima at $\alpha = 0$ and $\alpha = \pi$ as per Figure 5. Within particles on the order of single domain size, a successful spin reversal only occurs when the thermal energy present can flip the majority of all moments present within a very short time (on the order of $10^{-9}$s or less) and force a new conformation $^97$.

**Figure 5:** (a) Schematic of a prolate spheroid depicting a nanoparticle with uniaxial magnetic anisotropy in the presence of an external magnetic field $\mathbf{H}$ at an angle $\theta$ relative to the direction of the anisotropy axis. Angles $\alpha$, $\varphi$ give the orientation the magnetization of the particle, $\mathbf{M}$, relative to the anisotropy axis and the magnetic field, respectively. (b) Magnetic orientational potential energy as a function of angle $\alpha$ in the absence of an applied field, solid line (---), and in the presence of an applied field along the anisotropy axis, broken line (-- --). The minima occur at $\alpha = 0$ and $\pi$. Figure and caption reproduced with permission from Papaefthymiou, G.C., Nanoparticle Magnetism. Nano Today, 4 (5), 438–447. $^96$

Thus, at any given instantaneous point in time, a superparamagnetic particle can be considered to be a highly magnetised sample of material. But observed over a sufficiently long period of time (substantially greater than $10^{-9}$ seconds), the particles should be observed to have zero net magnetisation $^97$. When an external field is applied, particles are driven towards a single anisotropy energy minima as per Figure 5 and so achieve observable magnetisation comparable to their bulk material. Superparamagnetic materials also do not aggregate as much as less fine magnetic materials, because of this lack of net magnetisation in the absence of field $^96$. This allows them to remain dispersed prior to magnetisation, which is relevant for their use in tuneable ferrofluids $^{98}$, or biotechnology $^{88}$.

Magnetic nanoparticles tend to rapidly align with applied magnetic fields, and so have very short T1 relaxation times $^99$. Accordingly their most prominent use in mainstream biotechnology is as Magnetic Resonance Imaging (MRI) contrast agents, where they serve
to modify the T1 and T2 relaxation times of surrounding tissue\textsuperscript{75,80,99–101}. In circulation \textit{in vivo}, they can accumulate in tumours using EPR, and their own magnetisation to the MRI’s field hastens the conformation of the surrounding tissue to the field, which allows for superior imaging\textsuperscript{102}. This also means that the inclusion of magnetic nanoparticles for purposes such as magnetofection can create a multimodal vehicle trackable using MRI techniques\textsuperscript{102,103}.

When analysing magnetic nanoparticles, extremely low or non-existent coercivity and remanence coupled with high saturation magnetisation are good indicators that superparamagnetism has been achieved. An ideal superparamagnetic nanoparticle would possess no coercivity or remanence at all, but this is not necessary in biotechnology practices\textsuperscript{104}.

\subsection*{5.2.1.3 Magnetometry by SQUID}

The measurement of magnetic fields is possible through a huge variety of means, but the most popular modern apparatus for sensitive measurements with low noise factors, such as might be suitable for laboratory work, are Superparamagnetic Quantum Interference Devices (SQUID). Rather than passing samples through conventional inductive coils, SQUID uses superconducting materials at low temperature to form a Josephson junction around the sample.

A pair of Josephson junctions are arranged as per Figure 6\textsuperscript{105}. A superconducting loop is split in two places by an insulator such that as per the Josephson effect, a “supercurrent” (flow of current in the absence of applied voltage) occurs due to tunnelling across the junction. When a magnetic flux is within the coil a screening current is generated to neutralise flux, and where this screening current exceeds the critical current for the Josephson junctions they function as conventional resistors and a voltage appears across the junction. When the flux increases beyond a half of the magnetic flux quantum ($\phi_0$) for the system, to between $\phi_0$ / 2 and $\phi_0$, rather than screening the flux, it is energetically favourable for the current to amplify it to $\phi_0$ as the flux in the superconducting loop must always be a multiple of $\phi_0$ for the system. This results in a change in direction of current induced within the loop. The oscillation of voltage within the superconducting system is used to record each change in flux intensity by $\phi_0$ / 2. By using arrays of coils, whether this change is an increase or decrease can be extrapolated.
SQUID magnetometry is a capable but not necessarily competitive method for detecting magnetic nanoparticles distributed within samples of live cells, but it is appropriate as a means of characterising the properties of nanoparticles prior to use in biotechnology applications.

5.2.2 Materials for Magnetic Nanoparticles

Iron oxides, particularly magnetite, are the most well-investigated materials with which to construct magnetic nanoparticles. They continue to be important in nanomaterial construction because of a combination of strong quantifiable magnetic properties (particularly saturation magnetisation), and good biocompatibility. Iron oxides have relatively high Curie temperatures, low cytotoxicity, and almost all multicellular organisms have some form of iron metabolism capable of gradually removing them from open circulation and thus preventing excessive accumulation of particles in cells or patients.

The use of other effective magnetic materials such as Mg, Fe, Co, Ni, Zn, Mn, Gd is examined on occasion, but these typically have much lower biocompatibility. Gold nanoparticles are used in many biotechnology applications due to their inert nature, but they are not compatible with as many means of stabilisation, and their magnetic properties leave them much better suited to use as MRI contrast agents and other monitoring applications than as receivers for magnetic force. Their longer term fate in vivo also shows some poorly understood patterns of degradation which is of concern for their toxicity.
Many biotechnology applications seek to combine favourable elements of both iron oxides and other magnetic bulk materials. Iron alloy mixtures such as FeCo, and ferrites such as CoFe₂O₄, MnFe₂O₄, as well as more complex ferrites such as Ni₀.65Zn₀.35Cu₀.1Fe₁.9O₄ are often explored to try and unify favourable magnetic properties of non-iron oxide materials while retaining the biocompatibility of iron oxides. It is also desirable to address the issues that magnetite has with oxidation. Magnetite nanoparticles gradually oxidise into γ-Hematite and α-Hematite, which have lower saturation magnetisation. Inert coatings for magnetite such as gold can create a vehicle with adequate biocompatibility and superior magnetisation due to the lack of oxidation.

5.2.2.1 Synthesis and Storage of Superparamagnetic Nanoparticles

Superparamagnetic nanoparticles can be produced through bottom up or top down synthetic methods, but it is difficult to prevent aggregation in magnetic materials lacking superparamagnetic properties. An approach from beneath the critical size for single domain behaviour is easier. The theory of burst nucleation for nanoparticle production states that the ideal synthetic conditions for consistent and monodisperse nanoparticle formation involve conditions that quickly exceed critical supersaturation of the monomer components which will form the nanoparticles, without allowing further seed formation afterwards. This can be performed by rapid introduction of reagents, such as in a disk reactor, or by rapidly increasing temperature to a critical point at which seeding and growth is initiated, usually by thermal decomposition of reagents.

Due to their extraordinarily high surface area to volume ratios, nanoparticles tend to agglomerate in order to reduce their surface energy. To ensure that the particles produced remain reasonably monodisperse, a surfactant is generally included in synthesis reactions to prevent separate particle seeds fusing with one another. Superparamagnetic particles generally have poor solubility in solvents with strong bonding forces such as in aqueous phases, which can result in fusing, but corrosion is also a concern. These issues are counteracted by attachment of a polymer or acid coating or surface engineering with an inert compound such as silica most commonly.

The long term storage of superparamagnetic nanoparticles, particularly in the aqueous phase, has challenges. While surface oxidation is typical during synthesis, SPIONs have been observed to oxidise further during storage. Agglomeration that begins through...
fusion will gradually accelerate due to the increasingly ferromagnetic character of agglomerates, which will eventually compromise the superparamagnetism of a sample. 118.

5.2.2.2 Biocompatibility of Magnetic Nanoparticles

Leaching of nanoparticle materials into the cell can result in toxicity due to increase of potentially poisonous metal ions in the cell for more exotic metals. Iron and titanium are generally quite biocompatible 119 by contrast to cobalt, manganese, or cadmium and other popular metal nanomaterials 120-122, as they have routes for metabolism in the cell. But even many metals which are generally considered extremely safe and non-toxic in bulk form still display cytotoxicity when in nanoparticle form. Gold 77,107 and silver 123 in particular are notable examples, wherein there is evidence for a size dependent cause of mitochondrial damage by uncoated gold nanoparticles 124 despite the bulk material being one of the most chemically inert metallic substances known.

The pathway for this cytotoxicity is for the most part believed to be oxidative stress, which is a consequence of peroxidase activity that iron oxide nanoparticles have been known to display, catalysing peroxide breakdown into hydroxyl radicals, a reactive oxygen species, at pH values of 7 or lower 125. They may also trigger interactions with cellular components, and the spontaneous adsorption of proteins onto nanoparticle surfaces 126. In physiological conditions, magnetic nanoparticles have been observed to degrade in size and form ferromagnetic chains, which may be a related phenomenon 78,107.

The coating that is applied to nanoparticles is also very important with respect to triggering oxidative stress and other sources of cytotoxicity in cells 127,128. It has been observed that cationic external coatings are more toxic inside the cell compartment than anionic or neutral external coats made of similar materials 129,130. This is unfortunate given that many applications in biotechnology require a cationic external surface, particularly to facilitate attachment to and entry through the cell membrane.

A large body of research exists focusing on the prevention of protein adhesion to nanoparticles. Preventing the formation of a ‘protein corona’ on nanoparticles is an avenue to make particles less interactive with cellular components, promote less severe immune response and clearance, and retain more capacity to leverage surface and magnetic moieties 131. This can be accomplished by fluorination 132 or PEGylation 110,116 of the coat most
notably. A challenge for these methods is often the sacrifice of aqueous stability \(^{56}\), and decreased uptake in target cells \(^{42}\) that comes as a consequence of ‘stealth’.

Given the undesirable characteristics of excessively chained or agglomerated particles, protocols utilising superparamagnetic nanoparticles should seek to minimise the exposure to strong external fields, or moderate external fields over an extended period of time, during synthesis and storage\(^ {71,101}\).

The risk of contamination of therapeutic with compounds in use during earlier stages of nanoparticle synthesis can be a cause for concern for the biocompatibility of particles. ‘Green chemistry’, or use of protocols designed with an intention of avoiding environmentally hazardous byproducts and reactants can be a suitable evading this potential issue \(^ {133}\).

### 5.2.2.3 Synthetic Routes for SPIONs

Production of magnetic nanoparticles by co-precipitation can be achieved from mixtures of iron salts with differing oxidation states to achieve the mixed oxidation state of iron in magnetite. These can be relatively common iron salts such as ferrous and ferric chlorides \(^ {117,134,135}\) provided with reducing agents, or salts which co-precipitate more forcefully, such as aceytlacetonates \(^ {89,111,114}\) which decompose at higher temperatures, fusing their respective iron cations.

Sol-gel synthetic methods have also been a common means of magnetic nanoparticle preparation \(^ {89,136}\). For magnetite synthesis via sol-gel, precursor iron salts are embedded within a gel, dried and then annealed at high temperatures (300 °C – 400 °C) within a vacuum \(^ {137}\), after which particles can be extracted from the gel. All SPION synthetic protocols observed in literature ultimately seem to focus on triggering a co-precipitation reaction with a relatively high activation energy, from the sol-gel method, to less common means of reaction control in the form of reverse micelles containing aqueous salts within oil as uniform sized reactors \(^ {138}\), to more novel techniques such as mechano-chemical stimulation, where kinetic energy is used to trigger particle formation \(^ {133}\).

However, magnetite nanoparticles also occur naturally through production by enzymatic mechanisms in magnetotactic bacteria and in some multicellular organisms \(^ {139,140}\). The isolation of these particles has been proven a feasible means of acquiring stable magnetite particles suitable for use in physiological environments \(^ {139,141}\).
5.2.2.4 Common Surface Coatings for SPIONs

Coatings of weak acids and polymers used independently in other biotechnology applications satisfy the requirements of stabilising nanoparticles and ensuring favourable interactions with cells. Thus, oleic acid, citric acid, dextran, PEG, PHEMA, and PGMA are used as attachments to SPION \(^{21,75,131}\). Another notable coating is DMSA which achieves a considerably anionic surface charge for nanoparticles \(^{89}\). PAA is a viable coating which can serve as a linker for more functional outer polymer coats such as PEI, creating a single structure vehicle that can complex with DNA \(^{136}\). Poly-L-lysine can be used similarly while also reducing inflammatory response and extending circulation time of particles \(^{79}\).

Synthesis of SPION in the presence of sufficient polymer of relatively low pKa typically results in successful coating \(^{131,142}\). Following this, ligand exchange can be conducted at temperature (80 °C or more) between different pH sensitive polymers to create a coating that offers effective stabilisation for the pH that ligand exchange is conducted at \(^{131}\).
5.3 Magnetofection

While the earliest work with magnetic therapeutics was more than a decade earlier, a study into gene therapy aided by magnetic fields was not published until 2000, when the term ‘magnetofection’ as a descriptor for magnetophoretically guided gene delivery was coined, and has caught on since.

Magnetofection is a powerful tool, but can add complexity to the factors affecting successful transfection outcomes. Beyond the existing factors of relevance in gene therapy, the following should be considered where feasible:

- The nature of the superparamagnetic magnetic nanoparticles in use.
- How the magnetic nanoparticles associate with the transfection agent.
- How the magnetic nanoparticles associate with the DNA plasmid chosen for delivery.
- The characteristics of the external magnetic field used.

Consequently, while magnetofection can be utilised as a technique to enhance most gene therapies, there is a cost to be paid in complexity for the benefits offered. This would suggest that magnetofection should be primarily developed for applications where it can achieve greatly improve transfection efficiency over alternatives.

5.3.1 Previous Effectiveness of Magnetofection

Effectively gauging the performance of magnetofection agents poses challenges, because the modification of transfection agents to accommodate magnetic moieties can have a negative impact on their effectiveness in the absence of magnetic field. As a result, not all literature ultimately display a ‘fair’ comparison between magnetofection agents and equivalent non-magnetic transfection agents. The improvement in overall DNA expression following treatment with magnetofection complexes in vitro in the presence of a magnetic field in published work has been significant, as much as 10-fold over similar non-magnetic protocols. However, depending on the agent and cell type, the loss of transfection activity from the complexation with magnetic moieties could be estimated to lie between 10% and 60%. This is presumably due to interference with the ideal electrostatic function of the agents, reduced flexibility, or increased size during cellular entry. That said, a recent study has reported that for some agents, complexation with magnetic nanoparticles could improve transfection even in the absence of a magnetic field, which
was believed to relate to increased sedimentation rates onto cells as well as superior protection from sources of DNA degradation within the cell.

Magnetofection with non-viral agents has limited use so far in clinical trial settings, being a subset of the relatively few current non-viral trials \(^{34}\). The delivery of DNA using magnetic particles was only employed in two clinical trials identified. A pair of Phase I clinical trials was conducted in cats for the delivery of plasmids intended to promote anti-tumour immune responses in felines with fibrosarcoma, using PEI coated SPIONs \(^{147,148}\). Treatment was found to be well tolerated: despite enzyme-linked immunosorbent assay (ELISA) noting expression within the subjects, treatment did not cause any of the side-effects that would be expected from elevated systemic expression in humans of an equivalent granulocyte-macrophage colony-stimulating factor. The efficacy of the magnetofection as an adjuvant to surgery was not confidently assessed in either study however.

Interest in the technique building, with publications on the topic tripling in number over 6 years \(^{21}\). While clinical use is an attractive goal for a prospective magnetofection agent, the overwhelming majority of magnetofection protocols must be assessed and compared in terms of their performance \textit{in vitro}. The overall transfection efficiency achieved by a magnetofection protocol is therefore significant, but even \textit{in vitro}, factors such as the role of magnetic field conditions can be examined prior to any \textit{in vivo} testing.

\textbf{5.3.1.1 Improved Transfection Efficiency}

For targets such as \textit{ex vivo} tissues and \textit{in vitro} cell lines, current magnetofection methods achieve good performance in excess of current non-magnetic, non-viral agents for moderately difficult to transfect cell lines such as osteoblasts (53\% efficiency as opposed to 21\% for non-magnetic protocols) \(^{149}\), mesenchymal stem cells (30 minutes magnetofection outperformed 6 hours of conventional transfection) \(^{150}\), and cortical neurons (48\% efficiency as opposed to 25\% for non-magnetic protocols) \(^{71}\). These studies also suggested that, as in non-magnetic transfection agents, increased transfection efficiency is associated with increased cytotoxicity, though not greatly so, as the reagents used (Polymag, nTMag) had low intrinsic toxicity.

The increase in performance can be extremely variable. Some cell lines and tissue samples already transfect with current non-magnetic agents at efficiencies greater than 90\% \(^{56}\) which
leaves little room for improvement. Nonetheless, for moderately difficult cell lines that display only 20%–30% transfection efficiency with Lipofectamine 2000, magnetofection agents have been observed achieving near double the transfection efficiency of best performing control reagents.\textsuperscript{17,71,151}

Kamau et al., in 2006, indicated that they were unable to achieve improved overall transfection with magnetofection when run to completion, but instead achieved competitive transfection efficiency over a shorter timeframe than was previously possible.\textsuperscript{42} It is worth noting that the many magnetofection studies following have not placed great emphasis on whether their non-magnetic transfection agents used in comparison are performing over timeframes appropriate for their use. While rapid transfection is desirable, it could be seen as misleading to report superior transfection performance within a 30 minute window in comparison to reagents best used \textit{in vitro} over 5 hours with little caveat, as was the case in some studies.\textsuperscript{152}

Many papers which observe effectiveness of oscillating field magnetofection over alternatives use relatively short transfection times, ranging from 30 minutes\textsuperscript{152} to 1 hour\textsuperscript{153}, to 2 hours\textsuperscript{154,155} to study magnetofection. While Lipofectamine 2000, the control reagent of choice, remains effective in these timeframes, they do not represent its best performance, with Fouriki et al. observing a 3-fold increase in the transfection activity of Lipofectamine 2000 in NCI-H292 cells between 2 hours and 6 hours.\textsuperscript{155} While establishing contrast with competing reagents, and demonstrating effectiveness over shorter timeframes is important, the fact that controls are operating well beneath saturation goes unremarked on. Establishing a standard of effective use of control reagents under best practice also seems important to set an informative upper bound on achievable transfection.

A magnetofection paper was examined that featured transfection exposure for 4 hours in serum free conditions, with Lipofectamine as a control treatment, in HEK293T cells, in keeping with conditions commonly used in this research.\textsuperscript{42} However, this paper consistently reports a transfection efficiency for Lipofectamine of 20% or less, in contrast with expected values from such conditions of 40%.\textsuperscript{156} Quantification methods for transfection from papers that assay protein expression, RNA silencing, or luminescence are much more challenging to compare than % transfection, as has been used in this research, with luciferase reporters for Lipofectamine 2000 giving results spread over 8 orders of magnitude between observed papers.\textsuperscript{154,155,157} Nonetheless, within these papers, Lipofectamine 2000 often performs exceptionally poorly compared to other reagents in
relatively easy to transfect cell lines over long transfection times, when Lipofectamine 2000’s standard of transfection in easy to transfect cell lines is sufficiently high that only the number of transcripts expressed per cell is likely to be increasing if so.

5.3.1.2 Static and Oscillating Modes

Early literature on magnetofection observed that placing cells exposed to a magnetofection complex upon a magnetic plate was sufficient to improve expression of delivered DNA plasmids. For this static mode of transfection wherein the magnetic field through cells was constant, significant gains in gene expression, between 1.5 fold and 10 fold, were observed. These improvements allowed for faster transfection, achieving a significant level of gene expression after only 20 minutes to 2 hours of exposure rather than several hours or even days as appropriate for non-magnetic agents.

More recently, it has been shown that oscillating the magnetic field at the target site both in vitro and in vivo can further amplify transfection activity. Oscillating magnet arrays adjacent to culture plates during transfection form the most common in vitro application of this technique, with magnetic particles coated with polymers previously effective for DNA encapsulation and delivery, such as PEI, being used to transfect neural stem cells, respiratory epithelial cells, or common model immortal cell lines such as HeLa.

The increased transfection effectiveness of an oscillating field over a stationary field was most pronounced at extremely low oscillation frequencies, on the order of 0.5 to 5 Hz, with differences of approximately 2 Hz sometimes producing a difference in transfection efficiency as high as 20%. This suggested that fine optimisation of field frequency may be critical. In some studies, the greatest enhancement of transfection efficiency was observed if the oscillation of the magnetic field was preceded by exposure to a static magnetic field for some time. Presumably this allowed vector particle accumulation onto the cells in the target area, as it does in approaches using only a static magnetic field.

Many of the studies which observed higher transfection activity in an oscillating magnetic field used a system of columns of permanent magnets which rotated around the sample and would thus impart magnet force upon multiple axes. Other studies used the commercially available nanoTherics Ltd. ‘Magnefect’ system, which provides considerable lateral oscillation of its permanent magnet apparatus. Under both systems, similar improvements were observed.
5.3.1.3 Targeting with Magnetic Force In Vivo

Substantive demonstrations of success in the use of magnetofection in vivo are rare in comparison to in vitro work. Studies reporting in vivo magnetofection outcomes often find that even for platforms that show vast improvements under magnetofection regimens in vitro and ex vitro, replicating these improvements inside whole animals may pose additional challenges. 51

Demonstration of targeted gene delivery activity in whole animals has been reported following directions derived from computer modelling, although the effectiveness of transfection outside the target magnetofection area was not decreased as much as hoped, with off target organs only receiving approximately half of an untargeted dose. 162 Notable in this case was the easily utilised delivery method of aerosolised magnetic nanoparticle – transfection agent – DNA complex solutions, non-magnetofection variants of which have been used in vivo for some time. 163 Recently there were successes in achieving high dose response transfection in spatial targets such as melanoma and neural tissue. Magnetofection with viral vectors was highly successful in achieving localised expression in vivo in skeletal muscle tissue targets. 38

Overall, magnetofection has positive prospects in achieving high transfection efficiency within a spatial target area in vivo. However, the fate of magnetofection agents does not seem to be so radically altered that passively targeted transfection in the liver and spleen for charged particles, or in lungs and intestine for uncharged particles, is beneath concern.

5.3.2 Magnetofection Agent Construction

The installation of magnetic moieties into effective transfection platforms can be accomplished by transient or irreversible processes. Transfection platforms can be attached directly to the shell of magnetic particles in lieu of their stabilising coat, through use of peptide linkers, or by relatively transient association through electrostatic forces. The synthetic methods used are varied, and even within different methods of association, it isn’t unreasonable to expect differing performance depending on the nature of the magnetic nanoparticles attached. 122

A recent study examined the role of common, relatively uncontrolled laboratory variables in synthesis (by iron sulfate coprecipitation using ammonia) and attachment protocols for
SPION, such as storage age of reagents used for synthesis, methods of distillation (and accordingly the eventual pH and molarity of water used), and small changes in temperature during low temperature co-precipitation methods. It was found that the differences in eventual efficacy of the particles as magnetofection agents in complexation with a PEI matrix were insignificant. The study did observe one factor of significance, the pH of water the particles were stored in post-synthesis. Nanoparticles stored in non-alkaline conditions resulted in less effective magnetofection agents, achieving a transfection efficiency of around 25% in murine melanoma cells in contrast to the 40% achieved by particles stored at pH 10. This resonates with oxidation and suspension stability issues generally observed with water phase superparamagnetic nanoparticles.

The schemes for magnetofection vehicle construction include synthesis of magnetic particles with the transfection agent already in place as the first stabilising coat, or seeding and growth of SPION within an entrapping matrix of the transfection agent. Ligand substitution can be used to replace an existing coating with the transfection agent, but this specific type of protocol is not seen commonly. A linker can be used to chemically attach the transfection agent to the coat of the particle. Lastly, charge interactions can be relied upon to self-assemble the particle, transfection agent, and DNA into a single complex, provided the charge ratios are managed effectively.

5.3.2.1 Synthesis In Situ

Previously, protocols have been designed that allowed the synthesis of superparamagnetic iron oxide nanoparticles directly stabilised by a PEI coat, and these have been shown to be high performing magnetofection agents. However, in the last decade synthetic methods have been developed for the synthesis of magnetic nanoparticles within large cationic dendrimers constructed from biocompatible polymers.

As a potential approach for magnetofection agent synthesis, this has both advantages and disadvantages. The dendrimer itself must be able to withstand the conditions that trigger the synthesis by co-precipitation of the nanoparticles, which imposes limits on the temperature and pH that can be used in synthetic protocols.

In this case, the PAMAM dendrimer was embedded with Ferrous chloride, and sodium borohydride was used as a reducing agent for precipitation. This offers potential for good monodispersity as the dendrimers limit the mobility of seed crystals, and the structure of
the dendrimers themselves should be analogous to unmodified dendrimers, allowing better comparison with previous, non-magnetic transfection agents. However, analysis of the particles showed a failure to generate superparamagnetic iron when 2nd generation and greater dendrons were used as reaction templates.

There is another novel mechanism available for synthesis of magnetic nanoparticles within suitable gene delivery agents in the form of a protocol for the preparation of lamellar magnetoliposomes. These lamellar particles were intended to achieve high resilience in vivo through the redundancy offered by their ‘onion’ like layers. Phosphatidylcholine based lamellar liposomes were seen to be able to generate iron nanoparticles internally from ferrous and ferric chloride mixtures over long time periods in relatively mild conditions (STP), and became responsive to magnetic fields.

5.3.2.2 Covalent Linkers

Where synthesis as a single platform is unsuitable, effective independently synthesised components can be linked. Pioneering magnetofection work conjugated adenoviral agents and magnetic nanoparticles using heparin sulfate. However, in non-viral platforms, particularly with respect to recent work, there is no need to use biologically active linkers.

Cationic dendrimers are often used to decorate SPIONs by this method. By functionalising nanoparticles initial coatings of SiO₂ with (3-aminopropyl)triethoxysilane to provide an open amino group, it was possible to synthesise successive generations of PAMAM dendron groups directly onto the nanoparticles. This produces a versatile biotechnology platform, and many novel magnetofection agents follow a similar scheme.

Linkers in gene delivery agent designs, as with linkers for other applications, should have satisfactory compatibility with ‘click’ chemistry methods where possible. Click chemistry relies on a statistical distribution of initiation sites that can be easily, rapidly and specifically modified, which allows for association of particle and transfection agent, or any other targeting moieties which may be in play, such as peptides and fluorinated groups. Click chemistry protocols in other biological applications can provide a library of suitably reliable reactions to quickly and reliably provide covalent links, such as copper catalysed alkyne-azide reactions.
5.3.2.3 Electrostatic Association

To stabilise magnetic nanoparticles in aqueous environments, their functionalised coating must be significantly charged, barring particles suspended by the steric forces of longer polymer chains alone, such as in dextran particles. As a result, this surface charge is often an available means to drive the association between the magnetic nanoparticles and the other components of the vehicle. DNA has a weakly negative external charge in water, and all non-viral transfection agents complex with DNA by having at least a portion responsible for DNA condensation localise a positive charge in water. As a result, by suspending magnetic nanoparticles with an anionic coat, they can also use this as a means of associating with transfection agents.

This type of association is fast and simple, but there are potential complications. The ratio of each of the three vehicle components to one another can have impact on the structure of the resulting complexes. In line with this, an excess of either of the anionic components will exceed the charge capacity of the cationic agent, resulting in inconsistent or simply insoluble complexes with relatively poorer transfection efficiency. The pH and molarity of solution are relevant, with certain salts in solution influencing which functional groups offer effective suspension. A NET surface charge for nanoparticles that is nearing neutral increases circulation time in vivo, but also reduces their rate of uptake by cells, which could pose problems for transfection.

5.3.3 Establishing a Mechanism for Magnetofection

Having established that utilising magnetic nanoparticles along with transfection agents that can complex with and deliver DNA can result in superior gene delivery in the presence of a magnetic field, it is necessary to question why or how this occurs. Several plausible mechanisms have been proposed, and cellular studies have been very good at identifying protein interactions that relate to the effectiveness and pathways through the cell used in magnetofection. Nonetheless, literature still does not present a complete picture of why magnetofection is effective.

Thus, while the contact and mechanosensitivity theories for magnetofection remain popular, it is possible that effectiveness is also driven by some mechanism downstream of endocytosis. It has been observed in older transfection studies that only 1 in 1000 naked DNA plasmids injected into cytosolic compartments are eventually
expressed \(^{170}\), which leaves plenty of capacity for improvement after the endocytotic stage of gene delivery by gene delivery agents.

More unusual means of magnetic sensing in organisms such as Faraday rotation, wherein the optical properties of materials are altered by magnetic polarisation, can be discarded in this case \(^{171,172}\). Many studies explore the endocytotic pathways in use, and given that they are well understood, magnetoreception by clathrins and other surface proteins would be expected to have been noted before now \(^{21,87,142,158}\).

5.3.3.1 Vector Sedimentation

It is often assumed in magnetofection literature that the primary cause of augmented transfection in static field magnetofection is the result of accelerated vector accumulation onto the cell surface \(^{21}\).

Only a small number of studies were found that provide insight into the particle dynamics of this mode of transfection enhancement \(^{158}\). Studies on magnetofection of \textit{ex vivo} respiratory epithelium tissues observe greatly improved magnetofection vector accumulation within cells under the effect of a magnetic field relative to a robust set of controls \(^{51,173}\). This is despite respiratory epithelial tissues having well developed mucociliary clearance mechanisms and a protective glycocalix coating that would normally greatly inhibit contact by high concentrations of transfection vectors.

Another possible means of increased cellular contact in magnetofection is that the motion of magnetic nanoparticles descending towards a horizontally oscillating magnet, as is the case in many magnetofection protocols, might cause the particles to pass through a wider cross-section of the area potentially occupied by target cells. Recently, simulations have been used to explore the possibility that oscillating magnetic fields can lead to more effective coverage of the zone of space inside a given well plate during magnetofection \(^{174}\). These simulations offer some support to the idea of improving coverage within the cell (See Figure 7), with a demonstration of potential for improved odds of hitting a cell sized target, typically on the order of 10 – 20 \(\mu m\) diameter.
Figure 7: The simulated projection of 50 nm magnetic nanoparticle trajectory on XZ–plane for a plate with magnets centred at 45 degrees from plate’s central vertical axis. In ‘a’ magnets rotate circularly around the plate with frequency 40 rpm (0.666Hz), in ‘b’ – magnet is motionless. 

It can be observed that the model particle’s horizontal oscillations are across a spiral of 250µm horizontal diameter for each interval of 250µm descended, and that the mobilisation of model particles towards the magnet array exceeds the rate of settling under gravity, albeit not by an order of magnitude. However, this is at a lower frequency, and higher particle mass, than many magnetofection protocols. This could imply that a typical magnetofection protocol would expect more thorough horizontal coverage for each interval of vertical movement than represented in the simulation’s model. The descent of the modelled particles under field covered one millimetre in just under 18 seconds.

This suggested that in a typical 24 well plate such as was used for in vitro testing in this project, similarly behaving particles should have achieved total sedimentation in well under 8 minutes, much less than the duration of a typical transfection or magnetofection protocol (which ranges from 30 minutes to several hours). Once magnetic particles with gene delivery complexes attached achieve contact with the cell however, there are other processes that may be relevant.

5.3.3.2 Endocytotic Passage

The immediately downstream process, that being the endocytotic entry into the cell, has been the subject of considerable scrutiny, with evidence that:
- Magnetofection does not allow for cellular entry in the absence of endocytosis \(^{158}\).
- Magnetofection is for the most part reliant on ATP mediated endocytotic processes \(^{87,158}\). However, magnetofection can proceed in the absence of clathrin, caveolin and lipid-raft mediated endocytosis specifically \(^{59}\). These may be cell line specific effects.
- Magnetofection cannot function during inhibited transport of endocytotic vesicles, suggesting that it does not offer any special means of endosomal transport or escape \(^{158}\).
- Superparamagnetic nanoparticles do remain within the cell cytoplasm (and do not achieve nuclear entry) following endosomal escape, and do not degrade on the timescales that transfection takes place over \(^{49,78,107}\).

A study examined the difference in performance of PEI based magnetofection agents with and without a magnetic field after the deposition by centrifugation of the agents onto the surface of HeLa, BEAS-2B, and HEP-G2 cells \(^{158}\). The improvements offered by a static magnetic field in this case were not significant. Overall, there is a great deal of literature support for the theory that there is little or no enhancement of transfection activity due to the presence of magnetic field after deposition of transfection complexes onto the exterior of cells.

5.3.3.3 Mechanosensitivity in Cellular Surfaces

NanoTherics Ltd. have proposed that the mechanism for the improvement of transfection efficiency in magnetofection with oscillating fields is promotion of endocytosis by the physical stimulation of the cell membrane. While the endocytotic pathways involved are well known \(^{161}\), their stimulation by this activity is not directly explored in any cited literature.

The basis for this belief appears to be a review article on the activation of cellular ion channels by conjugated magnetic nanoparticles induced to motion by a magnetic field \(^{175}\). This article made comparisons of a number of prior studies where lateral magnetic force applied by particles to the cell membrane was capable of triggering activation of many mechanically sensitive surface receptors such as integrins and calcium channels \(^{176,177}\). Therefore, a complex bound to a magnetic particle oscillated in multiple directions by external magnetic field may cause a more immediate endocytotic process to occur than would be typical for vectors concentrated on the cell surface by a static magnetic field.
A concerningly rare consideration in magnetofection papers was the possibility that factors of magnetofection apparatus other than the magnetic field of the oscillating magnet array contribute to the improved transfection efficiency of cells. One examined paper from 2008 took into account concerns about heating effects induced by electromagnets during an oscillating field magnetofection. However, it is more difficult to assess another potential systematic factor, vibrations from the motors present in oscillating magnet arrays, on the quality of transfection.

5.3.3.4 The Fate of Magnetic Materials Within Cells

Magnetic nanomaterials that are used in biotechnology are typically constructed and coated with an intent to delay their degradation for as long as possible. This prevents the cell from experiencing an excessive concentration of dissolved iron. Highly effective inert coatings seem to reduce the rate of formation of reactive oxygen species on nanoparticle surfaces as well. The end result however is that magnetised nanoparticle aggregates remain a structural feature within cells, and the exocytotic mechanisms that would clear the cell interior of nanoparticles used in biotechnology are decreasingly effective as the hydrodynamic size of nanoparticles increases.

There is evidence that over the long term, magnetic particles that did not fully degrade can cause long chain structures by coupling magnetically within cells, that remain observable in cells months after exposure. The implications of these nanostructures for cytotoxicity and for the health of whole animals and humans is still not fully clear. However, given the relatively short timeline of transfection, it is very questionable that they could effect the efficiency of gene expression following magnetofection.

5.3.4 Previous Optimal Conditions for Oscillating Magnetofection

The use of oscillating magnet arrays in magnetofection, and indeed the use of magnetofection at all as opposed to non-magnetic transfection techniques, brings in a new host of attendant variables as discussed in 5.3. The magnetic field strength, direction, and the change in these variables as a function of time would all need to be considered to predict the motions of superparamagnetic nanoparticle complexes in suspension and on the surface of cells. Assuming these motions are relevant to enhancing transfection, all these variables bare at least some scrutiny in creating the optimal scenario for the
magnetically aided delivery of DNA bearing complexes into cells. Therefore, this project drew on observations of the relationship between transfection efficiency and magnetic field variables from previous studies to inform best practise.

5.3.4.1 The Role of Frequency and Rotational Axes

It has been shown extensively that oscillating the magnetic field used in magnetofection around the target site can further amplify transfection activity both *in vitro* and *in vivo*. This effect is most pronounced at frequencies on the order of 0.5 to 5 Hz (Figure 8).

![Figure 8: A Representation of the range of oscillating magnetic field frequencies believed to contain the maximum of transfection efficiency in magnetofection studies where field frequency was varied. The maximum optimal frequency refers to the frequency displaying best transfection performance, and the minimum optimal frequency refers to any tested frequency one increment lower within the study than the best performing frequency tested.](image)

It is not known why these frequencies produce the best amplification in transfection. It could be speculated that these frequencies may provoked the highest response from mechanosensitive proteins on the cell surface linked to endocytosis, as per previous studies on activating transport proteins on cellular surfaces using the procession of attached magnetic nanoparticles. These frequencies did not necessarily appear to correspond readily to the frequency response for any of the bulk mechanical properties of animal cells.

The role of rotational axes for oscillating magnetofection has not been the subject of any study examined. However, of the papers examined within Figure 8 there was agreement in ideal frequency between papers utilising commercial linear oscillating magnet array
apparatus\textsuperscript{151,152}, lab-built linear oscillating magnet array apparatus\textsuperscript{160}, and circular oscillating magnet array apparatus\textsuperscript{153,154}. This suggested that during magnetic field optimisation, deviation from a best performance close to 2Hz would be surprising, and it is a suitable starting point for any conventional magnetofection systems.

5.3.4.2 The Role of Field Amplitude and Strength

Variables in oscillating field amplitude have previously seen scrutiny as a means of altering the quality of magnetofection. One study examined the role of variable oscillating field amplitude for a magnetofection apparatus with 2Hz frequency in the efficacy of PolyMag transfection reagent, where data indicated significantly improved transfection at extremely low amplitudes on the order of 200µm to 10µm\textsuperscript{154}. This is in keeping with recommendations for the nanoTherics Ltd. Magnefect system, another oscillating magnet array for magnetofection. However, the control values present in each plate vary significantly even after normalisation from a half dozen separate plates, which casts some doubt on the effects observed.

A more recent study into variations in the amplitude of oscillations during magnetofection reached a similar conclusion\textsuperscript{181}. Unfortunately, once again a relatively low effect size was observed, with significant outcomes limited to increasing the eventual expression per cell rather than the number of cells transfected. From these two studies we concluded that the ideal amplitude for linearly oscillating magnet arrays in magnetofection applications is around 200µm.

Field strength is a less explored variable in magnetofection, with only a few comparisons available between fields of differing intensity. A study examining the magnet to cell distances and thus field strength of a Magnefect system to amplify transfection of mucoepidermoid carcinoma cells by PolyMag magnetofection agent observed a saturation in magnetofection effectiveness at 40 mT field strength\textsuperscript{155}. Earlier studies investigating magnetic drug targeting suggest that a field of this magnitude would be able to cause efficient deposition of vehicles containing 50% magnetite by weight \textit{in vivo} at flow rates typical for capillaries, but not in arterial vessels\textsuperscript{182,183}. Magnetic fields at the cell layer of plates installed in a magnet array are recommended to operate with a field strength of between 70 and 250 mT, as well as having a field gradient of between 50 and 130 Tm\textsuperscript{-1} as part of a review of magnetofection protocols by Nature\textsuperscript{117}. These values closely match common magnetic array types\textsuperscript{42,164}, but other studies have suggested that cells more rapidly
endocytose lone SPION particles under much stronger fields than these values would indicate, and are not in danger of adverse reactions to field intensities up to 100x greater.

The role of magnetic nanoparticle selection in magnetofection agent design, which would effect biocompatibility and magnetophoretic force, is not well explored in literature seen. The overwhelming majority of studies in gene delivery by magnetic particle make use of similar formulations of SPIONs between 3 nm and 50 nm in size, lacking the diversity seen in other magnetic nanoparticle applications in biotechnology. Despite this relative conformity, what precisely it is that SPIONs bring to a magnetofection complex bears consideration.
5.4 Summary

Magnetofection is an emerging technology and while some mechanisms associated with its function are yet to be fully elucidated, it has known efficacy. Non-viral vehicles for gene delivery can be made suitable for magnetofection by the inclusion of magnetic nanoparticles, usually SPION. In the case of a static magnetic field, SPIONs and associated complexes carrying DNA are sedimented onto cells, improving the number of particles that achieve contact with cells.

When this magnetic field is oscillated, transfection efficiency can increase, which has been speculated to relate to the stimulation of mechanosensitive proteins, or improved contact with less than fully confluent cells. Studies seemed to suggest that for an oscillating field magnetofection application, a field with an intensity on target of greater than 70mT, a gradient on target of greater than 50 Tm⁻¹, and a linear oscillation at approximately 2Hz across a 200µm amplitude is an ideal starting points for any optimisation.

Magnetic nanoparticles should be examined via SQUID before and after coating modifications to control for changes to their suitability during synthesis, and their toxicity should be tested prior to use in magnetofection. In conjunction with transfection agents possessing up to moderate cytotoxicity, off-target toxic effects can be minimised in vitro through effective targeting.
6 Materials and Methods

6.1 Superparamagnetic Nanoparticles

6.1.1 Standard SPION Synthesis for this Project

6.1.1.1 Initial Synthesis

SPION synthesis was based off of a technique for the production of 6 nm magnetite nanoparticles described previously in Sun et al. 2004\textsuperscript{112}. This consisted of creating a suitable mixture of oleic acid (6 mmol), oleylamine (6 mmol), 1,2 Tetradecanediol, (10 mmol), and Iron (III) Acetylacetonate (2 mmol), suspended in a volume of benzyl ether (20mL). All reagents were purchased from Sigma-Aldrich. All glassware was flushed with nitrogen under heat prior to the addition of reactants to minimise free oxygen.

The mixture was heated to 200 °C (taking approximately 40 minutes), and it was sustained there for a further 80 minutes. Following this time, heating equipment was set to reflux the mixture at a target temperature of 300 °C. Upon reaching the target temperature (requiring approximately 15 minutes), the reaction mixture was sustained at that temperature for exactly 1 hour before all heating elements were disabled and insulation was removed, to allow the reaction to cool to room temperature.

Following the reaction, to wash the product, the reaction mixture was removed from the vessel and magnetic stirrer and thoroughly combined with 60mL of ethanol to precipitate. Centrifugation at 200 g x 10 minutes was used to precipitate the magnetite particles. Supernatant was removed, and the particles were resuspended in a small volume of hexane (~5mL) with trace oleic acid and oleylamine added (Around 0.1% by volume). The particles were centrifuged again at 2000 g x 10 minutes. The supernatant was retained and any pellet disposed of, before reprecipitating the nanoparticles with more ethanol (generally requiring around 12 times the volume of hexane). After further centrifugation at 2000 g x 10 minutes and resuspension into around 20mL of hexane, the particles were considered washed and ready for characterisation or other uses.

6.1.1.2 Coating

For protocols where water solubilised SPION were required, citric acid coating was performed. This protocol was based on the example provided for citric acid coating iron oxide nanoparticles described previously by Lattuada & Hatton 2007\textsuperscript{131}. Nanoparticles
were precipitated and dried from the previous synthetic route, and suspended into a thoroughly stirred 1:1 mixture of 1,2 dichlorobenzene, and N,N dimethylformamide, 15mL of solvent for every 100 mg of SPION particles.

Citric acid was added to equal the weight of SPION present. This addition was followed by stirring the reaction mixture under heat to 100 °C, for 24 hours. After the reaction mixture has cooled, ethyl ether of 4.5 times the solvent volume is added to precipitate the mixture.

The precipitated SPION was recovered from the solution by use of a magnet, and then resuspended into Acetone and transferred to centrifuge tubes. The SPIONs were centrifuged at 500 g x 10 minutes and resuspended into fresh acetone 4 times to wash away remaining reactants. Finally, they were resuspended into 10mL of water with a few drops of NaOH solution added, centrifuged at 100 g x 2 minutes, and the solution along with any still suspended particles were considered the final yield.

A typical yield in practice was 70 mg of water soluble particles from the initial input of 100 mg of particles when citric acid was added 1:1. C-SPION was stored in aliquots containing approximately 25 mg /mL particles, with NaOH added to achieve a pH of ~10 to prolong effective life in storage.

6.1.2 Alternative Metal Nanoparticle Synthesis

In addition to the standard protocol for SPION synthesis above, other synthetic sources of magnetic nanoparticles were explored.

6.1.2.1 Cobalt Ferrite Nanoparticle Synthesis

In addition to the synthetic route for SPION production outlined earlier, Sun et al. 2004 also contained information on variations of this synthetic technique capable of producing CoFe$_2$O$_4$ based nanoparticles instead of Fe$_3$O$_4$ particles. They are also cited as being produced at slightly greater effective size, with 10 nm cores instead of 6 nm in the case of SPION. Considerably resembling the previous method:

All glassware, was dried in an oven at 50°C, prior to being set in place. The stirring equipment was also treated this way. The glassware and stirring equipment was further dried using cycles of exposure to vacuum and point heating to 200 °C under Ar.
$\text{Fe(acac)}_3$ (0.75 mmol, 0.265g) and $\text{Co(acac)}_2$ (0.5 mmol, 0.129g) were weighed out and placed into the reaction flask. 1,2-tetradecanediol (1.5 mmol, 0.346g), oleylamine (5 mmol, 1.7ml), oleic acid (5 mmol, 1.6ml) were added to the reaction mixture. All these reagents were bought from Sigma-Aldrich.

The reaction mixture was degassed for 20 minutes at room temperature using a gas mixture of Argon (93%) and Hydrogen (7%). The reaction was heated to 100°C and kept at this temperature for 10 minutes. Thereafter, the reaction temperature was raised to 300°C and the mixture was refluxed for 120 minutes before cooling down to room temperature by removing the heat source.

Hereafter, the product was handled in air. The product was collected and dissolved in hexane (10ml) and precipitated using absolute ethanol (40 ml). The product was washed three times using mixture of hexane and absolute ethanol (10 ml hexane and 40 ml ethanol) and finally dispersed in hexane.

6.1.3 Quality Assurance of SPION

SPION yields for the above methods were characterised several ways. Freeze drying techniques were used to quantify the concentration of particles in suspension. SPIONs were also freeze dried and packed into sample holders for magnetometry in a superparamagnetic quantum interference device (SQUID) to estimate the conditions of the core metallic particles following coating.

6.1.3.1 SQUID Magnetometry

SQUID Magnetometry followed a standard battery of tests. The first was a zero field cooled curve measurement from 2°K to 350°K, wherein the particles were cooled to 2°K under a minimum of external magnetic field (The Earth’s magnetic field can be difficult to isolate samples from and as high as 0.65 Oe under some circumstances), then a 100 Oe external field was applied, and DC measurements of the magnetisation of the sample were made as the temperature of the sample slowly increased. The second test in the battery was a field cooled curve measurement, wherein the sample was magnetised by an external field of 70,000 Oe at 350°K, followed by the same protocol of cooling to 2°K under zero applied field and the heating towards 350°K under 100 Oe applied field while taking DC measurements of the magnetisation of the sample.
The third test was a 5\(^\circ\)K hysteresis loop where at a static temperature of 5\(^\circ\)K, the magnetic field applied to the sample was slowly varied from 70000 Oe, down to -70000 Oe, and then back to the original 70000 Oe, while monitoring the sample magnetisation. The fourth and final test was a 300\(^\circ\)K hysteresis loop where at a static temperature of 300\(^\circ\)K, the magnetic field applied to the sample was slowly varied from 70000 Oe, down to -70000 Oe, and then back to the original 70000 Oe, while monitoring the sample magnetisation.

Before subsequent data analysis, the diamagnetic behaviour of the sample, which is contributed to overwhelmingly by the sample holder’s own diamagnetic field, must be accounted for. The linear trend of the outermost sections of the 5\(^\circ\)K and 300\(^\circ\)K loops is isolated and subtracted from the overall dataset to accomplish this.

The subsequent analyses of these 4 tests can be used to make a number of observations about the nature of the magnetic particle samples collected. As the applied field magnitude is increased at 300K, near room temperature, the maximum magnetisation seen in the samples defines the “saturation magnetisation” ($\sigma_s$). The ideal product, magnetite nanoparticles exhibiting superparamagnetism, should possess a high Curie temperature, coupled with low to absent coercivity and remanence, and saturation magnetisation approaching the literature values for similar sized magnetite particles, which would be in the vicinity of 60 – 80 emu/g of Magnetite\(^{185}\).

### 6.2 Synthesis of Novel Transfection Agents

#### 6.2.1 PEI-PGMA Nanospheres

PEI-PGMA nanospheres for use in this project were synthesised according to a protocol published previously in Evans et al. 2011\(^\text{59}\), with fluorination of PEI conducted according to Lv et al. 2015\(^\text{39}\). All reagents utilised were bought from Sigma-Aldrich. In summary:

- PGMA was acquired through radical polymerisation from glycidyl methacrylate in methyl ether ketone (MEK) at 45°C using azobisisobutyronitrile as the initiator, followed by several intervals of washing by precipitation in diethyl ether. The batch utilised was determined by gel permeation chromatography (GPC) to have a molecular weight of 223 kDa and a PDI of 2.89.

- PEI was acquired directly as linear 1.2 kDa or 25kDa branched PEIs. However, a fluorinated branched PEI was prepared from the 25kDa branched PEI following a
synthetic protocol for the production of PEI with ~127 fluoroalkyl groups per PEI, each containing 7 fluorine groups. PEI was freeze dried in vacuum to remove water before dissolution in de-gassed, dried methanol under argon. Heptafluorobutyric anhydride and then triethylamine were added to the reaction mixture. The target molar ratio during synthesis in this case was 1 PEI : 134 heptafluorobutyric anhydride : 161 triethylamine, based on an assumption of PEI consisting of 50 monomer units each featuring 4 primary amine groups. 20mL of methanol for every 0.1g of PEI present was needed for adequate dispersion. After stirring at room temperature for 48 hours, the reaction mixture was dialysed against 1L of water 5 times, then freeze dried to acquire the product.

- 100 mg of PGMA and 20 mg of Rhodamine-B in 20mL of MEK were heated to a gentle reflux for 18 hours under positive pressure of N₂. Afterwards, evaporation at 45°C under vacuum was used to reduce the volume present to approximately 2mL prior to precipitation with 20mL diethyl ether, followed by resuspension in MEK and 3 iterations of washing to remove unbound Rhodamine-B.

- 6mL of a 1:3 mixture of CHCl₃ and MEK was prepared and kept agitated as an organic phase solvent for the next step of the reaction. PGMA-RhB (75mg) and oleic acid coated SPION (20mg) were dissolved in this organic phase, which was then added dropwise to 30mL of a 1.25% w/v aqueous solution of Pluronic F-108, followed by emulsion for approximately 1 minute in an ultrasonic bath. Following this, organic solvents were allowed to slowly evaporate from the pluronic solution overnight under a nitrogen flow. The reaction solution was collected and centrifuged at 2000G to remove excessively large or unstable aggregates of SPION and PGMA.

- The supernatant from the previous step was combined with PEI to make up a 50% weight aqueous solution of PEI (Containing 100 mg PEI). Dissolution of PEI in water for this step required several hours. Once all components were mixed, the reaction vessel was heated to 80°C for 18 hours. Nanoparticles were purified from the reaction solution by passage through an activated magnetic retention column followed by two washes with distilled water, then the column was demagnetised, and particles were collected by flushing with water. Aliquots were collected in 1mL Eppendorf tubes for quantification by lyophilisation, and, due to concerns about
degradation over time of PEIs fluorinated by this anhydride reaction identified in previous papers \cite{39}, buffered to a pH of 5.5 with ethanoic acid prior to storage.

6.2.2 Dendronised Polymer

Dendronised polymers were received from a batch already quantified and published previously in Kretzmann et al. 2017 \cite{56}. The material, consisting of a co-polymer backbone statistically comprised of 92% HEMA and 8% GMA, grafted with 5th generation PAMAM dendrons appears in source literature denoted as ‘10b’ \cite{56}. A summary of the synthetic protocols necessary for their synthesis is recounted:

- The initiator for the atom transfer chain radical polymerisation (ATRP) reaction used to produce the polymer backbone was synthesized by reacting a 1:1:1 molar ratio of 4-(2-hydroxyethyl)morpholine with 2-bromoisobutyryl bromide and triethylamine at 0.229 mol/L each in dry toluene for 48h at ambient temperature. The resulting reaction solution was filtered to remove the amine salt, stirred with activated carbon, dried with MgSO4, and filtered, and the solvent was removed by vacuum distillation.

- The ATRP reaction to produce the backbones began with 1:3 monomer:methanol solutions of 3.6 mmol GMA, 29.0 mmol HEMA thoroughly degassed with nitrogen, with ATRP inhibitors removed by a basic alumina column, making up a total of 16mL monomer solution. The monomers were combined with 0.7 mmol CuBr, and 2.5 mmol of 2,2'-bipyridine in a reaction vessel. The morpholine based ATRP initiator was added as 210µL, 1 mmol, and the vessel was heated to 80 °C under nitrogen for 2 hours. After this time, 15mL of methanol was added and the reaction opened to air. Collection of product followed under reduced pressure to redissolve in minimal methanol, and reprecipitation steps in diethyl ether with centrifugation were used for washing, prior to the final drying of product by vacuum. The copolymer backbone was quantified by GPC to possess a molecular weight of ~15.7 kDa, and a PDI of 1.21. Proton Nuclear Magnetic Resonance (1H NMR) was used to verify the 8% GMA composition.

- Backbones were azido functionalised with 1.5g of copolymer (containing 0.82 mmol epoxides) in 30mL dimethylformamide (DMF), and stirred with Sodium azide (45 eq) and ammonium chloride (41 eq). The reaction occurred at 60 °C for
72h. Backbones were washed through reprecipitation and redissolving in diethyl ether and DMF respectively with centrifugation, prior to being dried in vacuum.

- Asymmetric propargyl-PAMAM Dendrons were generated as per a previous paper \(^{186}\), where an iterative 2-step sequence was used. Propargylamine was used as a starting point, with each PAMAM polymer generation built up in methanol first by methyl acrylate (3.5 eq), then after the removal of excess reactants, subsequently completed by the addition of excess ethylene diamine. Both reactions were conducted over 4 hours at room temperature.

- 4.5 generation propargyl-PAMAM dendrons (2.4 eq) were attached by dissolution in DMF (15 mL) before the addition of azido-functionalised copolymer (200mg). Both species were dissolved before the addition of pentamethyldiethylene triamine (PMDETA, 0.11 mmol). The reaction solution was degassed and backfilled with argon, before the reaction commenced under argon with the addition of CuBr (0.11 mmol). Reaction proceeded over 72h at room temperature before being dialysed against distilled water (4 × 4 L) and product collected via lyophilisation.

- This product was re-dissolved in minimal MeOH and added dropwise to a solution of excess ethylene diamine at 0 °C; reaction was left to proceed at room temperature for 7 days, then diluted with water and dialysed against distilled water (4 x 4L) before being lyophilised to afford a white solid, with completed PAMAM dendrons up to 5\(^{th}\) generation.

6.3 Cell Culture

6.3.1 Care and Passage of Cells

Two immortalised cell lines were used for \textit{in vitro} testing during experiments, MCF-7, and HEK293T, kept in T-75 flasks until use. MCF-7 cells were maintained in 12mL MEM-\(\alpha\) with 10% FBS, HEPES buffer and non-essential amino acids. HEK293T cells were maintained in 12mL D-MEM with GlutaMAX and 10% Foetal Bovine Serum (FBS) (Gibco, Invitrogen). Cell culture products were Gibco™ brand from Life Technologies Australia Pty Ltd. Cells were kept incubating at 37 °C in a humidified 5% CO\(_2\) atmosphere by a HERACELL VIOS 160i incubator system.
Cells were monitored for growth and at 75% confluence were passaged or prepared for plating by removal of media, washing with 2mL phosphate buffered saline solution, after which the phosphate buffered saline was replaced with 2mL 0.25% Trypsin-EDTA. Cells were returned to the incubator to trypsinise for 10 minutes, after which the cells were collected with an additional 4mL of appropriate full serum media and pelleted by centrifugation at 1000g for 2 minutes.

Cell pellets were resuspended from pellets in 10mL of full serum media, and mixed by pipette. 2mL of cell suspension along with 10mL of full serum media was used to complete the passage into a flask.

For each experiment requiring plated cells, two samples of 10 µL of cell suspensions were counted using a haemocytometer to determine cell concentration for plating. The cell suspension was then diluted further with full serum media. MCF-7 Cells were plated at a target 177,000 cells/mL, and HEK293T cells were plated at 200,000 cells/mL. In 24 well plates, 500 µL was used per well.

The tube containing the cell suspension was mixed by inversion before commencing each distinct plate, and mixed by pipette before withdrawing the mixture for each distinct well. Following plating, well plates were agitated side to side to ensure even settlement.

6.4 Transfection Protocols

6.4.1 General Transfection Protocol

During transfection experiments, variants of the following general protocol were performed.

18 - 24 hours prior to transfection, cells were plated in 24 well plates in appropriate full serum media. In the case of HEK293 cells, well plates were exposed to poly-L-lysine for 5 minutes, and then dried for 2 hours prior to plating. Wells were designated for at least 3 distinct replicates per condition per plate, with each plate being designated to a distinct magnetic condition. In the event that 3 or more plates were used, only one positive and negative control well per plate was included, otherwise the controls took place in triplicate on each plate.

Following plating, cells were placed into incubation until transfection. At least 30 minutes prior to experiment, reagents were removed to room temperature.
The following working stocks were used during experiments utilising 36 wells or fewer:

- DNA Stock: 210 µL (100 ng/µL) EGFP pDNA mixed with 490 µL OptiMEM
- LF2000 Stock: 21 µL Lipofectamine-2000 mixed with 680 µL OptiMEM
- nTMag+ Stock: 30 µL nTMag+ along mixed with 60 µL of Sterile filtered MiliQ water. nTMag+ should be mixed thoroughly, but subsequently allowed to settle once at room temperature, prior to sampling.
- For PEI-PGMA Nanospheres, working stock was 200 µg / mL in MiliQ water.
- For the novel dendronised polymer, working stock was 9 mg/mL in MiliQ water.
- For citric acid coated SPION, working stock was 5.45 mg/mL in MiliQ water.

0.6mL Eppendorf tubes were prepared for each well. 18 µL LF2000 stock was added to each tube. Where novel dendronised polymer was used, 2.88 µL of stock was applied per well to align with the optimal N/P ratio for the agent. At this point, timing commenced and 18 µL DNA Stock was added to the well tubes for the first plate in line for treatment. DNA was added under the meniscus and mixed by pipette thoroughly.

4.95 µL of nTMag+ stock was added to the surface of the fluid in the tube was added at this point, for a 1x nTMag+ treatment. Where citric acid coated SPION were used, stock would be applied at the appropriate quantity at this point.

At 20 minutes on the experiment timer, additional MiliQ water was added to tubes to equalise volumes towards 54 µL per well.

At 25 minutes on the experiment timer, the cell plate was removed from the incubator, and for each row in turn, media was removed and replaced with ~150 µL per well of PBS. Then, this PBS was removed and replaced with an equivalent volume of new PBS, then this second wash was removed. Each well was provided with 200 µL of OptiMEM. In each case this was with the well plate tilted 45 degrees, and the pipette tip against the side of the well plate facing downward.

At 30 minutes on the experiment timer, 50 µL of each experimental mixture was added to the respective wells dropwise. Sham wells did not receive any treatment at this point. Plates were immediately returned to the incubator for the duration of transfection: In alignment with the Magnefect with its oscillations activated where relevant.
Once the transfection timeframe concluded, well plates were removed from the incubator, and OptiMEM was removed gently from one row at a time, replaced by 500 µL supplemented full serum media. Plates were examined by microscopy and underwent quantitative analysis as close to the 48.5 hour mark on their transfection timer as was feasible.

For qualitative analysis, all populated wells utilised in transfection experiments were examined by epifluorescence microscopy before proceeding to quantitative measures. Wells with outlying levels of cell confluence or death were excluded from quantification as part of this process. Images taken at this stage allowed for examination of transfection distribution amongst cell populations, as well as lipoplex distribution where DiI allowed it.

6.4.2 Cell Survival Assay
A CellTiter-Glo® Luminescent Cell Viability Assay kit from Promega Corporation of Fitchberg, WIS, USA was used to assess relative cell viability. Cells were plated into 125 µL within a 96 well plate for the experiment. Cells were exposed to reagents as per a transfection protocol, but with all quantities at one quarter those utilised in a 24 well experiment. Following the 48 hour incubation period, the standard protocol for the kit was followed to prepare and apply the CellTiter-Glo reagent to cells.

6.4.3 DiI Staining
1,1'-Dioctadecyl-3,3,3',3'-Tetramethyindocarbocyanine Perchlorate, common name “DiI”, was acquired from Life Technologies Australia Pty Ltd. Of Mulgrave, VIC, Australia, for use as a lipid binding stain.

Several transfection protocols called for DiI staining of Lipofectamine 2000. DiI was initially prepared by suspension and dissolution of the dry salt in ethanol by sonication, first at 2.5 mg/mL, then undergoing 100-fold dilution in yet more ethanol as per Invitrogen™’s standard protocol to create a working stock. A staining protocol that did not rely on centrifugation was employed wherein Lipofectamine 2000 stock was mixed with 0.025 mg/mL DiI solution in ethanol at a ratio of 10:1. This mixture was left at room temperature for at least 2 hours prior to use in transfection, after which it was diluted to a Lipofectamine 2000 working stock as per section 6.4.1.
6.4.4 Statistics

Significance of outcomes was identified where relevant using GraphPad Prism 6 to conduct analysis of variance (ANOVA) of data with an α value of 0.05, with Tukey’s multiple comparisons test used to curtail “p-fishing” \(^{187}\). Prism 6 also recognises further significance cutoffs at \(p < 0.01\), \(p < 0.001\), and \(p < 0.0001\). Mean effect size is stated where relevant.

6.5 Analytic Devices

6.5.1.1 Superparamagnetic Quantum Interference Device (SQUID)

Magnetometry by SQUID was performed on a Magnetic Property Measurement System 3 (MPMS3) by Quantum Design, of San Diego, CA, USA.

During sample preparation, polypropylene P125E VSM Powder Sample Holders were used as primary sample containment, which were in turn placed within a P125A Brass Half-Tube Sample holder. These were also purchased from Quantum Design, of San Diego, CA, USA.

6.5.1.2 Electron Microscopy

Electron microscopy imaging was performed at the UWA Physics node of the Centre for Microscopy, Characterisation and Analysis in Perth, WA, Australia. The JEOL2100 Transmission Electron Microscope (TEM) used to image samples was operated at a beam current of 68A, and an accelerating voltage of 120kV for imaging.

Samples were deposited onto 200 mesh copper grids, unadorned for cell sections. In the case of nanoparticle imaging, a 30 nm Formvar resin and 5 nm amorphous carbon coating was present to provide physical support, as per ProSciTech item GSCu200C.

6.5.1.3 Dynamic Light Scattering

Dynamic Light Scattering (DLS) techniques to assess both the hydrodynamic size and zeta potential of complexes was performed on a Malvern Instruments Zeta Sizer Nano ZS system, which utilises a 4 mW He-Ne laser operating at 633nm with a scattering angle of 173°. 500 \(\mu\)L suspensions of nanoparticles were allowed to complex for 25 minutes at the
Effective concentrations used for complexing prior to in vitro treatments, and placed in a disposable folded capillary cell for repeated measurements of hydrodynamic size and zeta potential at 25°C. This test battery was conducted in triplicate over 15 minutes to control for time dependent instabilities observed in some reagents relevant to this thesis.

Calibration was conducted with ‘dispersant’ defined as water at 25 °C (refractive index at 1.33 and viscosity 0.887), and in most cases defining the ‘material’ as the magnetite present (refractive index 2.42, absorbance 0.01), however in assessing PGMA nanospheres, PGMA values were used (refractive index 1.515, absorbance 0.05). Zeta potentials were measured at pH ≈ 7.

6.5.1.4 Epifluorescence Microscopy

Bright field and epifluorescence microscopy imaging was performed on an Olympus IX-51. The Green Fluorescent Protein (GFP) fluorescence signals were acquired using the U-MGFPHQ (excitation between 460 and 480 nm, emission between 495 and 540 nm) filter set. Red Fluorescent Protein (RFP) and red fluorophore signals (DiI and Rhodamine B, two fluorophores used in this thesis have, very similar excitation and emission to RFP) were acquired using the U-MRFPHQ (Excitation between 535 and 555 nm, Emission between 570 and 625 nm) filter set.

6.5.1.5 Flow Cytometry

Flow cytometry assessments were performed on instruments available at the Harry Perkins Institute of Medical Research node of the Centre for Microscopy, Characterisation and Analysis. Flow Cytometry data reported here was acquired using an BD LSRFortessa. For GFP detection, an excitation and emission wavelength of 488 nm and 530 nm respectively, was used. For detection of red fluorescent proteins such as those produced by expression of the mCherry reporter plasmid, or red fluorophores such as DiI, the excitation and emission wavelengths used were 561 nm and 610 nm respectively.

Cells were collected for flow cytometry from 24 well plates following growth by removing media from the wells, washing each well with 200 µL of phosphate buffered saline, then replacing with 150 µL of trypsin-EDTA. Cells were returned to the incubator to trypsinise for 10 minutes, then each well was collected along with an additional 300 µL of full serum media into a labelled eppendorf tube. Cells were pelleted by centrifugation at 500 g for 2
minutes, and the pellets resuspended by pipetting into 500 mL of a FACS buffer consisting of phosphate buffered saline with 1mM EDTA and 2% FBS added.

6.5.1.6 Inductively Coupled Plasma / Mass Spectroscopy (ICP-MS)

Inductively coupled plasma analysis of acid digested samples for iron determination was conducted using an Agilent Technologies 5100 ICP-OES.

Iron content was assessed by acid digestion of representative samples in 7.5M Nitric acid at 95°C in a sand bath for 1 hour, followed by resuspended in a 1M nitric acid buffer for ICP-MS to gauge iron content. Samples were run alongside a TraceCERT 1000 mg/L standard of iron in dilute nitric acid, diluted to 200ppm, 20ppm, and 2ppm in 1M nitric acid buffer.
7 Results & Discussion

The sequence of analyses used to understand the development and effective use of each prospective magnetofection agent in turn was made from the following sequence where applicable.

- Magnetometry
- DLS of Complex to observe stability and DNA binding
- Early transfection to ensure effective use
- Comparative transfection to optimise magnetic field
- Epifluorescence microscopy to visualise transfection outcomes
- Flow cytometry to quantify transfection outcomes.

With the exception of SPIONs which were shared between prospective agent designs and are thus covered immediately, this section is oriented around this sequence of values for each prospective agent in turn.
7.1 SPION

The first necessity for production of transfection agents with magnetic moieties was the preparation and analysis of the magnetic moieties themselves. Under examination by TEM, synthesised particles were not highly monodisperse, in contrast to images from the source of the protocol in Sun et al. 2004\textsuperscript{112}, with a distribution of core diameters between approximately 6 nm and 18 nm as can be seen in Figure 9. The typically observed size of around 10 nm was larger than the intended core particle size of 6 nm.

![Figure 9: A TEM micrograph displaying citric acid coated SPION dried from a dilution in distilled water to concentrations used in complexing, around 0.11 mg/mL, for verification of individual particle size, and metallic crystal structure within particles. Image was taken using JEOL2100.](image-url)
Aliquots of citric acid coated SPION were digested for analysis by ICP in order to quantify the iron concentration of particles after coating substitution.

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<th>UNIT</th>
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</tbody>
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Table 1: Sample quantification of citric acid coated SPION by ICP. A 44.5 mg sample of citrate coated iron nanoparticles was digested and resuspended in 10mL, and diluted further to a 1 in 10 solution.

ICP-MS indicates ~15.75 mg of iron present in the 44.5 mg sample of nanoparticles as per Table 1. Assuming particles conform to a formula of Fe₃O₄, Fe comprises 167.541 / 231.537 or ~72.4% of the ferrite mass present within the nanoparticles is represented in the weight of iron. Therefore we can assume that there is ~21.75 mg of magnetite in the 44.5 mg sample, or ~48.9% of the SPION sample is ferrite. It was assumed that this proportion is relatively consistent for fully stable citrate coated nanoparticles, as another aliquot from a separate, earlier synthesis digested for analysis presented 39.1% iron by weight and therefore 54% magnetite by weight in SPION. This would suggest that this synthetic method was not perfectly consistent in output, but nonetheless produced particles with approximately 50% of their weight made up by their SPION core, which allows for easy comparison with magnetophoretic mobilities observed and modelled using sample 50% Fe₃O₄ nanoparticles in previous papers.¹⁷⁴,¹⁸²,¹⁸₃,¹⁸₈
7.1.1 SQUID

7.1.1.1 Oleate SPION

Figure 10: Representative SQUID Magnetometry data collected from oleic acid coated SPION particles following synthesis, dried from hexane.

A: A 5 °K Loop adjusted to the weight of nanoparticles present, and with the trend from diamagnetic moment removed.

B: A 300 °K Loop adjusted to the weight of nanoparticles present, and with the trend from diamagnetic moment removed. Inset bottom right: a magnified view of low field interactions relevant to the particles’ hysteresis.

C: Field cooled curves adjusted to the weight of nanoparticles present.
7.1.1.2 Citrate SPION

Figure 11: Representative SQUID magnetometry data collected from citric acid coated SPION particles following ligand exchange, collected by lyophilisation.

A: A 5 °K Loop adjusted to the weight of nanoparticles present, and with the trend from diamagnetic moment removed.

B: A 300 °K Loop adjusted to the weight of nanoparticles present, and with the trend from diamagnetic moment removed. Inset bottom right: a magnified view of low field interactions relevant to the particles’ hysteresis.

C: Field cooled curves adjusted to the weight of nanoparticles present.
7.1.1.3 SPION Magnetic Values

As Figure 10 and Figure 11 indicate, the extent of measurable coercivity and remanence at 300 °K was not large, under closer examination they were observed to be around 28.5 Oe and around 1.3 emu/g respectively after ligand exchange, but were slightly higher beforehand at around 30 Oe and 2.5 emu/g. This small difference may be due to the additional washing and filtering steps involved in ligand exchange further selecting away from the aggregations responsible for the ferromagnetic character of the sample. The field cooled curves (Figure 10c and Figure 11c) would appear to support this notion, with the lower and narrower observed blocking temperature of around 50 °K as opposed to 100 °K following coating being consistent with results expected from SPIONs of a smaller average core size. Coercivity and remanence were observed to be around 70 Oe and 4.2 emu/g seen at 5 °K, indicating that the reduction of the effective hysteresis of particles by superparamagnetism when over their blocking temperature is noticeable, but not as great as would be seen in large superparamagnetic particles from literature of sizes above 20 nm.

Both coercivity and remanence are often reported in Tesla to satisfy requirements for reporting in SI and to allow comparison with bulk materials. This would provide a 300 °K coercivity of 2.85 mT, which is effectively negligible when compared to literature values for bulk ferrite materials, and consistent in magnitude with literature values for ‘superparamagnetic’ iron oxide nanoparticles, only somewhat above typically published ‘superparamagnetic’ values of around 1.7 mT.

As for remanence, comparison to literature is more challenging as studies of bulk materials typically also report this in Tesla, which requires a volume based rather than mass based susceptibility to be known. Previous papers have demonstrated that assigning a volume susceptibility based on the ideal volume of ferrite crystal present based on the mass of iron is considered adequate. Thus, referring to the figure of 48.9% magnetite present in SPIONs derived in Section 6.2.3, and the theoretical density of magnetite of 5.2 g/cm³, we can:

- Consider the magnetite present to have a remanence of around 2.66 emu/g.
- Consider it to therefore possess an ideal volume susceptibility of around 0.511 emu/cm³
- Report volume susceptibility as an SI unit measurement of remanence equal to 0.642 mT.
This is again, negligible in comparison to bulk ferrites, and appropriate for an iron oxide nanoparticle \(^{101,190}\). Overall, these SPIONs could be considered to have relatively poor quality superparamagnetic behaviour, being on the upper boundary of the criteria used in literature, but nonetheless seem appropriate to be referred to as a superparamagnetic iron oxide nanoparticle, with an observable 300 °K hysteresis less severe than any reported ferromagnetic iron oxide nanoparticles in literature. It is notable that Sun et al. 2004 did not display 300 °K hysteresis values of lesser magnitude than those observed here when deciding their superparamagnetic criterion \(^{112}\). Given the extremely low but non-zero coercivity observed, it would be expected that the particles produced consist of SPION with a relatively small number of emergently ferromagnetic particles present.

Accompanying minimal hysteresis at 300K, particles had a strong saturation magnetisation of 43 emu/g of particles (\(~\)88 emu/g of ferrite, \(~\)121.5 emu/g of iron) that could be maintained throughout ligand exchange processes. These figures compare to typical values for superparamagnetic nanoparticles and indeed to those of bulk magnetite in general \(^{185}\).

### 7.1.2 DLS and Zeta

Citric acid coated SPION were to be added directly to aqueous environments to complex electrostatically with cationic reagents as part of some transfection agent synthesis protocols. Their behaviour alone was observed in a PBS environment to model their behaviour in physiological conditions. In PBS, C-SPION particles were observed with a hydrodynamic size by intensity of 39nm, with a PDI of 0.4, the citric acid coating was able to provide a zeta potential for particles between -20.6 mV and -23.0 mV (95% CI).

While this hydrodynamic size by intensity is quite large compared to expectations, the hydrodynamic size computed by number had a very tightly clustered distribution around 15.7 nm, with 10% of particles detected having hydrodynamic size greater than 20nm. This warrants comparison to the intended SPION core size of 6nm, the typical observed SPION core sizes of around 10nm, and the largest observed SPION core sizes of around 18nm. It is therefore reasonable to assume that in physiological conditions such as in the application of magnetofection agents in this thesis, the existence or formation of relatively small clusters of 10 or fewer SPIONs are a typical occurrence, but larger aggregations towards micrometre scales were not observed within the \(~\)30 minute complexation times monitored by DLS.
7.2 PEI-PGMA Nanospheres

PEI coated PGMA nanospheres containing SPION, were synthesised as per Evans et al. 2011 and explored as potential magnetofection platforms, and a schematic can be seen in Figure 4. It was expected that the PEI coated PGMA nanospheres, having demonstrated good cellular entry and drug carrying profiles, would be effective at delivering DNA under the effect of a magnetic field.

Several different PEI’s were used as coatings, a linear PEI of 1.2 kDa, a branched, 25kDa PEI, and a 25kDa branched PEI fluorinated as per a technique which has improved transfection outcomes previously. While fluorinated PEI was expected to have high performance as a serum-resistant transfection moiety, investigation following synthesis revealed a poor stability in water, which made it more difficult to work with, hindering its analysis and diminishing its promise somewhat.

7.2.1 DLS and Zeta

Initial optimisation steps through DLS looked for vehicle : DNA ratios at which the PEI-PGMA nanospheres could achieve consistent polyplex formation for small differences in polyplex contents, which was considered a good indicator of the suitability of formulations for in vitro work. At near-even ratios, measured hydrodynamic size and zeta potential for complexes altered significantly over small changes in ratio or over repetition, eventually becoming more stable at ratios featuring less DNA, as per Figure 12, Figure 13 and Figure 14. These ratios with stable complex production were considered more useful for in vitro work.
Figure 12: Zeta potential and hydrodynamic size by intensity data collected from preparations of branched PEI-PGMA nanoparticles with EGFP plasmid DNA in PBS. Each data point represents triplicate examination of two physical replicates. Error bars display SD.

Figure 13: Zeta potential and hydrodynamic size by intensity data collected from preparations of linear PEI-PGMA nanoparticles with EGFP plasmid DNA in PBS. Each data point represents triplicate examination of two physical replicates. Error bars display SD.
As displayed in Figure 12, Figure 13, and Figure 14, size and zeta potential were variable until Nanoparticle : DNA ratios reached 12:1 or greater. Therefore, transfections proceeded on the assumption that Nanoparticle : DNA ratios between 12 and 20 were appropriate for this class of particle. It can be observed that the nanoparticles with a linear 1.2kDa PEI coat were ultimately able to achieve the greatest zeta potentials in the presence of plasmid DNA.

The vehicle : DNA ratio identified would not outcompete existing high performance transfection vehicles, as Lipofectamine 2000 for instance ideally operates at vector : cargo ratios in the vicinity of 3:1 to 6:1 by mass \(^{191}\). It may be that this related to the effective surface area of the PGMA Nanospheres, as the DNA / particle complexes were around 140 nm at their most compact, and while Lipofectamine 2000 complexes have much larger hydrodynamic diameters in the vicinity of 1 micron \(^{192}\), this represents lipofectamine assembled around condensed DNA, so they may be able to pack more efficiently, while the PEI-PGMA nanospheres remained separate until relatively high quantities of DNA were present, which de-stabilised their zeta potential.

The PEI coated PGMA nanospheres here display a maximum zeta potential of around 30mV, seen in Figure 13, in contrast to literature values, which exceed 50 mV \(^{59}\). While this raised concerns of poorer levels of PEI attachment than seen in literature, it was also likely
to relate to the use of PBS as a medium during measurement, which would be expected to mask the effective surface charge of particles, in contrast to the literature value which was measured in water. It also notable that the average hydrodynamic size was larger, at around 200 nm rather than 110 nm as seen previously. Given the difference in measurement medium, and the role of sonication in achieving this particle size during synthesis, this was considered an acceptable deviation.

7.2.2 Epifluorescence Microscopy

Following attempts to utilise the PEI-PGMA nanospheres in transfection, epifluorescence microscopy was only able to verify a minimum of transfection activity. Transfection activity by linear PEI-PGMA nanospheres was observed at around the same order of magnitude as the rate of uptake as free EGFP plasmid (several individual cells per well, which considering the effects of growth would imply a transfection efficiency of around 0.0001%). Fluorinated branched PEI-PGMA nanospheres were more effective, but still only resulted in extremely low levels of plasmid expression, comparable to subsequent wells assessed by flow cytometry at around 2% effective transfection, an example of which can be seen in the quarter well image in Figure 15b. The PEI-PGMA nanospheres as per their construction in literature 59 were stained with Rhodamine B, a fluorophore which could be monitored using epifluorescence microscopy. Through this red fluorophore signal (As per Figure 15c, Figure 15d) it could be clearly seen that particles were being uptaken at high rates in all cells despite only a small number of cells ultimately achieving expression of the delivered plasmid.
7.2.3 Discussion of PEI-PGMA Nanospheres

Red fluorescence signal received from cell imaging verifies the literature observation that nanospheres of this design could be uptaken efficiently by cells \( \textit{in vitro} \) even in the absence of a magnetic field \(^{59}\). Nonetheless, given the nature of the results, PEI-PGMA nanospheres at the vehicle : cargo ratios utilised clearly did not deliver DNA effectively as part of this uptake process.

The later addition of a static and oscillating field during transfection could have improved transfection efficiency. However, given that particles could be internalised effectively at a high rate over the transfection time involved in literature \(^{59}\) and in these experiments as per Figure 15c and d, and magnetofection is not believed to assist transfection beyond the point of internalisation \(^{49,158}\), this did not hold good prospects for improvement on the orders of magnitude necessary for competitive transfection outcomes. In the pursuit of
highly effective platforms for magnetically aided gene delivery, this did not constitute a promising starting point.

Clearly there was interaction between DNA and nanospheres as evidenced from DLS, but it may be that DNA could not be condensed onto the nanosphere surface sufficiently to protect it from degradation within endosomes as free PEI would be capable of. An alternative or possibly complementary explanation is that previous literature observed that PEI coated PGMA nanospheres absent of DNA did not exhibit evidence of being capable of achieving endosomal escape at a high rate. Observations of the localisation of Rhodamine B signal in cells (See Figure 15d) seemed to indicate localisation within vesicles rather than free signal within the cytoplasm, which would support this explanation. It is worth noting that Tangudu et al. in 2015, when examining PEI coated PGMA particles also noted particle uptake rates of 95%, similar to what can be observed in Figure 15c and d, but were additionally able to achieve effective RNA silencing in a variety of cell lines by using the particles to deliver interfering RNA vectors.

While it is only necessary for the considerably smaller DNA cargo to escape the endosome for successful expression, if the PEI-PGMA nanospheres used as vehicles were unable to compromise endosomes, the DNA would not escape. It seems likely that the vehicles generated were not able to utilise their PEI coating as a proton sponge at a sufficient level to allow for the endosomal escape of DNA and release of their encapsulated DNA plasmids into the cell. This could have been due to particles having a lesser level of PEI attachment during synthesis than would have been ideal, but there is not an apparent explanation as to why this would occur.

Fluorinated PEIs have been able to complete transfection with lower vehicle : cargo ratios than unfluorinated PEIs in previous studies. This could suggest that in this case, the quantity of PEI present on the surface of PGMA nanospheres was closer to adequate for endosomal escape in the case of fluorinated PEIs, and so some effective transfection was achieved. Ultimately, in view of poor performance, further development of PEI-PGMA nanospheres as magnetofection agents was halted.

7.3 Novel Dendronised Co-Polymers / C-SPION

Another previously published novel transfection agent that was examined as a prospective magnetofection agent was a dendrimer consisting of 5th generation poly(amido amine)
PAMAM dendrons distributed across a statistical co-polymer backbone of 92% HEMA and 8% GMA \(^5\)\(^6\), and a schematic can be seen in Figure 3. This dendrimer was cationic and was reported to possess a zeta potential of around 45 mV in water. Therefore, it was expected to able to complex with both the anionic SPIONs and DNA electrostatically, which would allow for vehicles to be assembled from these components at the time of transfection.

7.3.1 DLS and Zeta

The dendronised polymer was examined prior to addition of magnetite. The hydrodynamic size by intensity in PBS when complexed with DNA at an N/P of 10 (The best performing ratio in literature) was observed to be approximately 180 nm with a PDI of 0.23, and the zeta potential for the polyplexes was observed to be between 13.7 mV and 15.5 mV (95% CI). While there is no DLS data provided in literature for this specific formulation, similar polymer formulations created polyplexes of approximately 180 nm hydrodynamic size, and 18 mV zeta potential \(^5\)\(^6\).

When these polyplexes of dendronised polymer and plasmid DNA were accompanied by citric acid coated SPION, changes were seen in the hydrodynamic size and zeta potential, as seen in Figure 16. The goal of DLS as an early optimisation step in this case was to determine the maximum quantity of magnetite that could accompany the dendronized polymer – plasmid DNA polyplexes without compromising the zeta potential of the vehicle to an unacceptable level (10 mV or less).
Figure 16: A graph of the hydrodynamic size and zeta potential of polyplexes observed with DLS of dendronized polymer and pDNA with an N/P of 10, along with citric acid coated SPION, in PBS, as the amount of SPION was decreased and thus the polymer : magnetite ratio was increased.

At near-even ratios as well as very high ratios, measured hydrodynamic size and zeta potential for complexes altered significantly over small intervals or with repetition. More consistent behaviours were observed in the 4 – 8 range of Polymer : Magnetite ratio. Reliable complex formation was considered important for transfection. In view of this data, it was concluded that polymer : magnetite ratios in the vicinity of 4:1 were appropriate for use in magnetofection.

7.3.2 Epifluorescence Microscopy

Epifluorescence Microscopy was able to verify the presence of significant transfection activity by the dendronised polymer when in the presence of citric acid coated SPION. An example of this can be seen in Figure 17. In light of this candidate magnetofection agent displaying effectiveness, quantification of transfection outcomes was undertaken.
Figure 17: An example bright field (A) and GFP fluorescence (B) image of HEK-293T cells transfected with an EGFP containing reporter plasmid by a 4:1 ratio of dendronised polymer : citric acid coated magnetite. Yellow scale bar is 100µm.

7.3.3 Flow Cytometry

Having established that the dendronised co-polymer / C-SPION complexes had effective transfection activity, their performance as magnetofection agents at a variety of magnetite concentrations in the vicinity of the earlier optimum ratio of 4 : 1, polymer : magnetite, was tested. A magnetic field appropriate for magnetofection use was also applied to understand the response of the magnetofection complex to field. The outcomes of this transfection were quantified by flow cytometry to provide fine distinction over the concentration range, shown in Figure 18.
Figure 18: The measured transfection efficiency of differing formulations of PAMAM dendronised HEMA/GMA co-polymer together with citric acid coated SPION, as well as controls, in delivering and triggering expression of EGFP plasmid in HEK293T cells after 4 hours of transfection and 44 hours growth. Bars labelled “2Hz Magnetic Field” were exposed to a 2Hz frequency x 0.22mm amplitude oscillating magnetic field during transfection. Each data point was harvested from 3 wells of a 24 well plate.

These results did not indicate a strong possibility of significant improvement in transfection outcomes using SPION in conjunction with the dendronised polymer, rather the opposite. Not only did the presence of citric acid coated SPIONs in the transfection solution reduce the effectiveness of the dendronised polymer as a gene delivery agent (p < 0.0001, ANOVA considering variance of all data as a function of Polymer : SPION ratio), the application of a magnetic field further lowered effectiveness (p < 0.05, ANOVA considering variance from magnetic field over all paired data). While the latter factor could be a consequence of differences in cell behaviour and treatment between plates, multiple plates were necessary to establish all non-magnetic and magnetic conditions in this instance, thus it is likely that this effect is real.

7.3.4 Discussion of Dendronised Polymer

The presence of citric acid coated SPION in the transfection mixture was seen to not only decrease transfection efficiency at higher concentrations, as expected, but also appeared for
the most part to negatively affect transfection outcomes; even further under the effect of an oscillating magnetic field, which is a poor indicator for a candidate magnetofection agent.

It is possible that the dendronised polymer / DNA complex responded poorly to sedimentation by magnetic field, such as having some irreversible interaction with the wellplate floor, or that polyplexes which incorporated SPION were less effective at condensing DNA or achieving endosomal escape. The clustering behaviours that might be expected of SPIONs brought close together as part of a complex, especially given that the particles used here were not perfectly superparamagnetic, and that the reduction in transfection intensified under the application of an external field, may have been had adverse effects on the conformation of the polyplex.

It is also possible that as in the case of subsequent experimentation, the more achievable objective in the use of magnetofection with the dendronised polymer would have been in achieving good transfection over a shorter timeframe rather than improvement of absolute transfection effectiveness. All dendronised polymer based transfections were conducted over a 4 hour exposure time for maximum transfection efficiency in order to align with the best practice established by previous literature pertaining to the use of the compound in gene delivery.  

With consideration of both the inability to achieve improvement through the addition of magnetic moieties to the dendronised polymers, and ongoing practical difficulties in working with the material, development of magnetofection agents based on this scheme was halted. While other dendronised polymer formulations achieved greater transfection efficiency in literature, the poor response to SPION association suggested a superior magnetofection agent was unlikely to develop from this formulation.

7.4 Lipofectamine 2000 / nTMag+

Lipofectamine 2000 is a gold standard non-viral transfection agent. While designs for magnetoliposomes exist, there was interest in the creation of a magnetofection scheme with agents as similar to the standard of Lipofectamine 2000 as feasible. This would allow for close and highly valid comparison of the effectiveness of transfection under magnetic and non-magnetic routes. Difficulty in establishing magnetic moieties offering good association with Lipofectamine 2000 ensued as the existing citric acid coated SPION and a
neutrally charged dextran coated SPION that was examined failed to offer a means of associating with lipoplexes without eliminating transfection activity. It was decided that a commercial magnetofection reagent would be ideal to examine. In order to more effectively monitor the behaviour of Lipofectamine 2000 liposomes during magnetofection, a lipophilic fluorescent dye, DiI was used.

Most commercial magnetofection reagents are marketed as single platforms that associate with DNA and drive transfection on their own, but nTMag Plus (or nTMag+) was a commercial magnetic moiety designed for magnetofection, intended for use in conjunction with existing effective transfection agents. The nTMag+ platform consists of a core SPION particle of around 10 nm, with a thick polymer coating, closely resembling an earlier product of the same design with independent transfection activity, nTMag. Transmission electron microscopy as per Figure 19 appears to support this.

Figure 19: A TEM micrograph displaying nTMag Plus dried from a dilution in distilled water to concentrations used in complexing, for verification of individual particle size, and metallic crystal structure within particles. Image was taken using JEOL2100.

Compared to SPION synthesised in the course of this experiment, the average core particle size of nTMag+ appears considerably lower. Particles were observed as small as 4.5 nm, and as large as 13 nm, with the typical size of particles being around 9 nm. The tight
clustering and chaining behaviours seen in TEM images like Figure 19 suggested that the polymer coating does not keep individual nTMag+ SPION cores separated while they are dry.

While other prominent nanoTherics Ltd. magnetofection agents such as nTMag and Neuromag are well explored in existing studies, little information is available publicly about nTMag+ and it has relatively little published utilisation. Accordingly, exploration of nTMag Plus’ effectiveness and best use is of value, and is presented here.

7.4.1 DLS and Zeta
DLS was ultimately not a feasible means of primary characterisation and early optimisation for the nTMag+ / Lipofectamine 2000 complex. Thorough analysis of all formulations by DLS techniques would have been wasteful of the limited material that was available, however analysis of the nTMag+ particles alone in PBS returned a hydrodynamic size by intensity of approximately 655 nm with a PDI of 0.3, and a zeta potential of approximately -24 mV. Modelling of the number distribution suggested that most particles present had a hydrodynamic size of around 140 nm.

The most closely related particle in the nanoTherics library, nTMag, has a particle diameter with coat included of approximately 100 nm \(^{169}\). In view of this, the DLS results suggest that nTMag+ particles, while likely similar individually, exhibited some aggregation even when stable in solution.

7.4.2 Epifluorescence Microscopy
Under epifluorescence microscopy, the level of EGFP expression achieved by nTMag+ associated lipoplexes was comparable to that of Lipofectamine 2000 without magnetic moieties, which placed it as a high performing formulation. Through this means, it was also determined that nTMag+ and DNA alone do not display any transfection activity in excess of naked plasmid, which contrasts with the effective independent magnetofection activity of its parent formulation, nTMag. In addition to the standard role of verifying effective transfection was achieved by cells, epifluorescence microscopy was particularly useful in tracking DiI tagged Liposomes used for transfection. See Figure 20.
Figure 20: An example of the fields which were examined to qualitatively verify transfection effectiveness in transfections using Lipofectamine 2000 based transfection reagents and EGFP plasmid prior to quantitative assessment. In this instance, MCF-7 cells in a 24 well plate were treated to a 4 hour transfection time with 500ng DNA complexed with 0.525μL Lipofectamine 2000 stained with 0.025μg of DiI. Cells were then allowed 44 hours of growth in full media before observation.

A: A bright field image which clearly displays both adherent cells and the plentiful non-adherent cells that arise from treatment with Lipofectamine 2000.

B: A fluorescence image to capture GFP fluorescence indicating the effectiveness with which delivered DNA was expressed.

C: An image of red fluorophores which displays clearly the uptake of DiI by cells, and by extension the ultimate fate of the liposome materials tagged with it, within the treated cells.

7.4.2.1 Verification of Effective DiI Staining of Lipofectamine 2000

Literature protocols for applying DiI to liposomes required the dye be applied in an aqueous environment at a high concentration, then removed with supernatant following centrifugation. However, Lipofectamine 2000 is highly stable in water, and centrifugal forces exceeding 20,000g would have been necessary to collect it in an efficient and effective fashion. Additionally, further characterisation would be necessary to verify full recovery of the Lipofectamine 2000. Instead, DiI staining proceeded without the removal of excess, free DiI. In view of the use of a DiI staining protocol diverging from previously published protocols, considerable scrutiny was applied to ensure any free DiI present did not compromise the effectiveness of other analytical methods.

Free DiI remaining in the DiI stained Lipofectamine 2000 stock was observable with epifluorescence microscopy, able to stain membranes when applied at concentrations relevant to transfection. However, it was not significant relative to the intensity of stain imparted to the Lipofectamine 2000 liposomes even at the lowest effectiveness observed. Cells were exposed to DiI solution at an effective concentration equivalent to 100% of the DiI used to stain Lipofectamine 2000 normally used for transfection (0.054 μL of DiI working stock) being present as free dye. It was found that in this case, as in cases where the DiI was bound to Lipofectamine 2000, fluorescence intensity increased following
further incubation, but in the case of free dye it still did not approach levels which would introduce significant noise in identifying cells containing stained liposomes, see Figure 21.
Figure 21: Fields displaying the relevance of staining of cells from free DiI throughout transfection time. The leftmost images display bright field images which clearly identify the cells within the field of view. The rightmost images display red fluorescence microscopy images indicating the DiI stained particles and cells within the field of view.

A, B depict MCF-7 cells exposed to free DiI equivalent to concentrations used in transfection, for 4 hours and imaged immediately.

C, D depict MCF-7 cells exposed to free DiI equivalent to concentrations used in transfection, for 4 hours, then washed and allowed to grow for a further 44 hours before imaging.

E, F depicts MCF-7 cells exposed to DiI stained Lipofectamine 2000 for 30 minutes prior to washing and imaging.
Using epifluorescence microscopy, the accumulation of DiI signal over time could be observed. Free DiI was visible as a background red fluorophore signal, and gradually stained the outer membranes of cells very weakly over the course of hours at relevant concentrations. Over the following 48 hours growth, this stain became localised to internal compartments. DiI stained lipoplexes were visible as small, very bright dots under epifluorescence microscopes and could be seen deposited rapidly onto the surface of cells and the well floor under magnetofection, or only associated with cells in conventional transfections (See Figure 21f). The staining of cells internally from dissemination of the DiI stain entering via lipoplexes progressed over hours to a pattern of both stained internal compartments, and a weak stain of the cytoplasm in general, and fluorophore intensity increased further over the course of several days.

7.4.2.2 Verification of Liposome distribution during Magnetofection
A significant change in the behaviour of liposomes during magnetofection could be visualised effectively using epifluorescence microscopy to examine the DiI fluorescence signal of tagged liposomes. As displayed in Figure 22, the use of magnetic moieties and an external oscillating magnetic field could deposit liposomes much more rapidly than occurred due to their free movement in the absence of field, but not so thoroughly as to eclipse the effectiveness of uptake over a longer transfection timespan.
Figure 22: Observations on the deposition of Lipofectamine 2000 during magnetofection through fluorescence microscopy of cells treated with DiI stained liposomes and then washed. The leftmost images display bright field images which clearly identify the cells within the field of view. The rightmost images display red fluorescence microscopy images indicating the DiI stained particles within the field of view.

A, B depict MCF-7 cells exposed to DiI stained Lipofectamine 2000 / EGFP Plasmid complexes for 30 minutes in the absence of a magnetic field.

C, D depict MCF-7 cells exposed to DiI stained Lipofectamine 2000 / EGFP Plasmid / nTMag Plus complexes for 30 minutes in the presence of a Magnefect system oscillating at 2Hz frequency, 0.22mm amplitude.
7.4.2.3 Verification of Co-Transfection Efficacy of MCF7 Cells

Co-transfection was used to contrast the efficacy of magnetofection and conventional transfection methods over small timeframes (Under 1 hour) and with challenging condition. Prior to quantification via flow cytometry, fields were captured using fluorescence microscopy to verify successful transfection and observe the distribution of transfection by each plasmid.

![Image of MCF7 cells with fluorescence microscopy](image)

In verification of co-transfection, epifluorescence microscopy offered utility in identifying the relative intensity of cells transfected with EGFP (495 nm – 540 nm emission), mCherry (570 nm – 625 nm emission), or both plasmids. Cells transfected by only one plasmid do not appear to display an obvious trend towards greater combined fluorescence intensity than co-transfected cells, which can be seen in Figure 23, suggesting that the Lipofectamine...
2000 / nTMag+ system did not exclude one plasmid from complexes in favour of the other.

7.4.3 Microplate Photometry
The use of microplate photometry was appropriate for gathering relative fluorescence intensity data in some experiments with the nTMag+ based vehicle. Microplate photometry was poor at distinguishing lower transfection efficiencies, but for protocols incorporating greater sources of fluorescence, and for high transfection efficiencies such as were being achieved according to microscopy, it offered a means of rapid whole plate analysis, and a gauge of expression levels rather than % transfected cells.

7.4.3.1 Verification of Effective Dil Staining of Lipofectamine 2000
A point of concern was whether Dil, or, more relevant given the widely accepted use of the fluorophore itself and the unusual protocol pursued here, the trace ethanol introduced to the transfection mixture, might have effects on transfection efficiency. This could occur by damaging cells or plasmids, reducing uptake and expression, or possibly increasing transfection by permeabilising cell membranes. The maximum ethanol concentration that cells could have been exposed to during transfection would be 0.017% w/v. While likely to be much less due to evaporation throughout preparation and transfection, this is a physiologically relevant concentration for some extremely ethanol sensitive cell receptors\textsuperscript{195}, even if it is below the threshold necessary to noticeably effect whole organisms such as humans\textsuperscript{196}. A transfection was conducted to examine the possibility of such an effect, with results displayed in Figure 24. Fluorescence from Dil in the transfected cells was beneath the detection limit of the photometer.
With all the above information considered, it was deemed that DiI staining via the protocol above was an effective and non-interfering method of monitoring Lipofectamine 2000 liposomes during transfection.

### 7.4.3.2 Cytotoxicity

Prior to the use of nTMag+ as a means of adding magnetofection capability to Lipofectamine 2000, it needed to be verified that nTMag+ did not have cytotoxic effects at the quantities used in transfection. Additionally, concern as to whether nTMag+ would interfere with the complexation process between Lipofectamine 2000 and DNA if added too early, needed to be addressed.
While all treated conditions are significantly ($p < 0.0001$) reduced compared to untreated control values in Figure 25, within the conditions treated with Lipofectamine 2000, there was not a significant difference between plates taken over all nTMag concentrations, and across all plates there was no difference among nTMag+ concentrations other than 5:1, which had significance ($p < 0.05$) relative to 1:5 and Lipofectamine 2000 only, the lowest concentrations of nTMag+. It can be concluded from Figure 25 that nTMag+, at the concentrations used in transfection, 2:1, may have minor cytotoxic effects in conjunction with Lipofectamine 2000, but the effect is small enough to be irrelevant next to cytotoxicity from Lipofectamine 2000 itself.

### 7.4.3.3 Effective Use of nTMag Plus

Three plates of cells underwent 4 hour transfections to examine the relative effectiveness of mixing nTMag+ with Lipofectamine 2000 prior to the addition of DNA (Pre-mixed) and after the addition of DNA (Post-mixed), in order to decide protocol for further...
experiments. Preceding further development on an effective method of utilising nTMag+ during transfection to prevent sedimentation (and thus utilising the nTMag+ mixed and cold, rather than according to the protocol described in methods), this transfection showed some cause for concern that nTMag+ can be detrimental to transfection efficiency under less than ideal complexing circumstances.

![Graph](image)

**Figure 26:** The GFP signal from MCF7 cells transfected in a 4-hour transfection with Lipofectamine 2000, followed by 44 hours growth. Cells were treated with either transfection mixture only, or with nTMag+ mixed either before or after the addition of the DNA to the mixture. The magnetic condition was also varied between no magnetic field, and unmoving magnetic field, and 2Hz x 0.22mm oscillating magnetic field. Each magnetic condition was performed on a separate plate. Each condition was conducted in triplicate and error bars display SD.

With all plates considered equally, there was not ultimately a detectable difference between pre-mixed and post-mixed to be observed. Post-mixed was used as the basis for subsequent experimental development as oscillating magnetic fields were of interest, however this may have been an arbitrary choice given confounding factors.

Transfections were conducted to observe the effect on the signal from expressed GFP protein when Lipofectamine 2000 was used with and without magnetofection over long and short periods.
Figure 27: The GFP signal from MCF7 cells after either a 30 minute, or 4 hour transfection with Lipofectamine 2000, followed by 44 hours growth. Cells were treated with either DiI stained Lipofectamine 2000 only, or with DiI stained Lipofectamine 2000 and nTMag. The magnetic condition was also varied between no magnetic field, and a field oscillating at 2Hz x 0.22mm using the Magnefect system. Each magnetic condition was performed on a separate plate. Each condition was conducted in triplicate and error bars display SD.

As shown in Figure 27, magnetofection using nTMag+ could significantly increase the effectiveness of transfection over a short timeframe, but was not able to achieve significant improvements over Lipofectamine 2000 during a much longer timeframe of transfection, and indeed was detrimental to overall expression in the absence of an oscillating magnetic field.

7.4.4 Flow Cytometry

7.4.4.1 Verification of Effective DiI Staining of Lipofectamine 2000

In order to gauge the likelihood of DiI staining being effective as part of flow cytometry protocols, wells exposed to 0.054 µL of free DiI for 4 hours were examined. Just under 5% of cells presented DiI stain satisfying the gating procedures established to distinguish cells with significant DiI signal as part of experiments using DiI stained Lipofectamine 2000. Accordingly, the maximum false positive rate for DiI stain during flow cytometry from the effects of free DiI can be assumed to be 5%.

Another potential confounding factor was that DiI, as a fluorescent agent, could potentially result in false positive signal in assessment of GFP due to spectral overlap. However, the spectral overlap of DiI and GFP expressed by cells was observed to be less than 0.5%, which could be compensated for easily. This supported the use of DiI as a marker in
concert with GFP fluorescence, as the maximum intensity of DiI signal observed from DiI stained liposomes and similar exposure to DiI was below the detection threshold of not only the microplate reader, but also the GFP detecting filters used in flow cytometry. Therefore, there was very little risk of DiI present in samples interfering with quantification of GFP as a reporter.

### 7.4.4.2 Magnetofection

Two transfections were examined under flow cytometry to observe the effect on the signal from expressed GFP protein when Lipofectamine 2000 was used with and without the magnetofection employing nTMag+ and a Magnefect system over long and short periods. As the proportion of cells DiI positive and proportion EGFP positive were monitored simultaneously, this also serves to demonstrate the effectiveness of DiI staining as a means of monitoring transfection efficiency. Figure 28 depicts the outcome of one such transfection, and Figure 29 depicts data from a separate transfection used for verification of effect, and reports coincident staining.
Figure 28: The percentage of MCF7 cells expressing significant DiI (A) or GFP (B) signal from a single experiment after either a 30 minute, or 4 hour transfection with Lipofectamine 2000, followed by 44 hours growth. Cells were treated with either DiI stained Lipofectamine 2000 only, or with DiI stained Lipofectamine 2000 and nTMag. The magnetic condition was also varied between no magnetic field, and a field oscillating at 2Hz x 0.22mm using the Magnefect system. Each magnetic condition was performed on a separate plate. Each condition was conducted in triplicate and error bars display SD.

Figure 29: The percentage of MCF7 cells expressing significant DiI and GFP signal simultaneously after either a 30 minute, or 4 hour transfection with Lipofectamine 2000, followed by 44 hours growth. Cells were treated with either DiI stained Lipofectamine 2000 only, or with DiI stained Lipofectamine 2000 and nTMag. The magnetic condition was also varied between no magnetic field, and a field oscillating at 2Hz x 0.22mm using the Magnefect system. Each magnetic condition was performed on a separate plate. Each condition was conducted in triplicate and error bars display SD.

The two separate experiments (Figure 28 and Figure 29), appeared to be consistent in outcome. In addition to the observable difference in transfection efficiency between the transfection efficiency of 30 minutes of Lipofectamine 2000 only and other conditions, there was no significant differences in transfection efficiency observed. Therefore, within
30 minutes of exposure, the addition of nTMag+ and an oscillating magnetic field was able to improve Lipofectamine 2000 transfection activity to a level indistinguishable with a 4 hour long transfection time.

There was a significant (p < 0.05) increase in the DiI uptake during 4 hour magnetofection over 4 hour conventional transfection. While the proportion of cells expressing DiI from liposomes was a relatively good predictor of transfection performance in this case, it seemed to be increased by the addition of nTMag+ to a greater degree than the eventual expression of GFP is, with GFP expression being the limiting factor in the signal observed in Figure 29. Overall, to a more exaggerated extent than observed using microplate photometry, magnetofection was a much more significant factor in improving the outcomes of transfections taking place over short timeframes.

7.4.4.3 Field Optimisation

Previous studies have overwhelmingly reported that a magnetic field oscillating at 2Hz frequency across an amplitude of 0.22mm is most effective for magnetofection. However, since the underlying reason for this is not elucidated at this time, good practice would dictate some examination of differing magnetic conditions with every course of experimentation.

A transfection (Figure 30) was conducted to observe the relative transfection efficiency of several variations on the assumed best oscillating magnetic field condition, 2Hz frequency with an amplitude of 0.22mm. As the proportion of cells DiI positive and proportion of cells EGFP positive were monitored simultaneously, this also served to demonstrate the efficacy of DiI staining as a means of monitoring transfection efficiency.
As per Figure 30, both in terms of the greatest transfection efficiency achieved overall, and the greatest increase in transfection efficiency under magnetofection, the 2 Hz x 0.22 mm condition appeared to be most competitive with other possible variations of oscillating magnetic treatment. However, the improvement in expression is not significant, save for the 2 Hz x 0.22 mm condition’s improvement over 3 Hz x 0.22 mm ($p < 0.01$), so it would be most accurate to state that this provides no cause to make accommodations for an optimal magnetic condition other than 2 Hz x 0.22 mm.
Of some concern is the unusually low transfection efficiency for all wells and plates during this experiment, which while almost precisely an order of magnitude lower than expected was confirmed in microscopy images captured at the time of measurement. Repetition gave similar results. Despite this, DiI staining indicates liposome uptake by a very large proportion of cells, comparable to expectations established by Figure 28a for a successful transfection. If it were expected that the DiI uptake is indicative of the optimal conditions for magnetofection complex uptake, there was no significant difference between fields and thus no cause to expect an optimal magnet condition other than 2 Hz x 0.22 mm.

7.4.4.4 Co-Transfection

Given that the greatest increases in transfection efficiency due to magnetofection were observed in challenging, adverse, or otherwise low-performing conditions for transfection in general, co-transfection, where the transfection vehicle is loaded with two different plasmids simultaneously (each at half the normal concentration during transfection), was considered.

![Graph showing transfection efficiency](image)

**Figure 31:** The percentage of MCF7 cells expressing significant RFP and GFP signal simultaneously after a 1 hour or 4 hour transfection, followed by 44 hours growth. Cells were treated with either DiI stained Lipofectamine 2000 and DNA only, or DiI stained Lipofectamine 2000 and nTMag as well as DNA, and exposed to a 2Hz x 0.22mm oscillating magnetic field using the Magnefect system. All conditions occurred on the same plate. Each condition was conducted in quadruplicate and error bars display SE.

As Figure 31 above shows, with the use of nTMag+ and magnetofection, Lipofectamine 2000 was able to achieve simultaneous expression of EGFP and mCherry plasmids, represented in a co-transfection efficiency equivalent to 4 hours of its conventional co-transfection, possibly better, with only 1 hour of magnetofection.
7.4.5 Discussion of nTMag Plus

While nTMag+ appears to utilise a core SPION, but in contrast to those synthesised according to schemes explored here, its observed ability to augment Lipofectamine 2000, an anionic liposome, as well as its negative zeta potential and advertised efficacy in concert with cationic polymers, suggest that its proprietary polymer coating may possess very distinct properties. It seems likely in view of this, and the acquired data, that it joins the transfection complex through some association with the DNA present rather than association with the transfection agent, as this would render it compatible with a wide variety of transfection agent designs.

7.4.5.1 Insights into the mechanism of magnetofection

The core SPION of nTMag+ was quite polydisperse with respect to the morphology and size, being similar to the SPION synthesised as per the Sun et al. 2004 protocol but of a smaller size. As it was ultimately an effective means of increasing transfection efficiency, it can be asserted that monodispersity was not a prerequisite for an effective magnetic moiety for use magnetofection in this case.

It is notable that during the magnetic field optimisation experiment (Figure 30), in comparison to the expression of GFP protein by cells with either transfection or magnetofection, the proportion of cells with uptake of DiI stain was much more similar between magnetic and non-magnetic condition, despite free DI, which would be unaffected by the travel of polyplexes, not being a significant factor. This was especially true in the case of poorly performing magnetic conditions. It seemingly stands opposed to the relatively weak trend observed in transfection experiments with either all the same magnetic conditions, or non-magnetic conditions, and over longer timeframes, where DiI uptake was seen to increase proportional to nTMag+ use and otherwise correlates with transfection. This raises interest in the idea that the nTMag+ lipoplex stability outside cells might relate in some way to the oscillating field frequency.

The data collected does not seem readily supportive of the theory that oscillating field magnetofection effectiveness is a property of mechanically sensitive surface proteins, as in that case it would expected that DiI would have substantially increased uptake in high performing conditions due to the facilitation of endocytosis by SPION oscillations during
the short transfection time. What can be observed in the magnetic optimisation experiment is if anything, the opposite of that (no significance was observed in the % DiI stain between magnetic conditions however), with the highest performing conditions failing to express DiI stain at a higher level than poorer performing conditions.

It is possible that this trend is the result of differences in relative health and survival of cells due to the concentrations of Lipofectamine 2000 and nTMag+ that they were exposed to during transfection. In this scenario, cells with lower exposures would replicate more rapidly following transfection and thus express their available transcripts of EGFP plasmid to a greater degree, but the survival assay conducted of nTMag+ and Lipofectamine 2000 in cells did not suggest a large difference in viability should be expected.

Another possibility is that nTMag+ does not only bind to Lipofectamine 2000 to affect its travel, as expected, but by itself mediates some form of interaction between cells and DNA, or lipoplexes and DNA. Lipofectamine 2000 is intended to allow passage directly across the cell membrane without DNA becoming sequestered within endosomes, and nTMag+’s affinity for DNA remains unknown. It could be that higher performing oscillation frequencies promote a dissociation of a nTMag+ and DNA complex into the cell at a critical point during cell attachment and result in the incorporation of only nTMag+ and DNA into cells while the liposomes fuse with the cell membrane to a lesser extent. Certainly, the related compound, nTMag, has efficacy as a transfection agent on its own, so if the two particles are sufficiently similar, nTMag+ may be facilitating some limited amount of cellular entry of DNA without liposomes involved. However, again, it is worth noting that nTMag+ is not advertised as having efficacy as a magnetofection agent in the absence of other compounds, and during speculative transfections it was not seen to cause any EGFP plasmid expression at all in the absence of any other transfection agents.

The lack of any disagreement on optimal magnetic field conditions with previous studies at the very least implies that the mechanism for the divergence between liposome uptake and DNA expression in the field optimisation experiment is unlikely to be a unique property of nTMag+. Given observations on the sedimentation pattern of DiI stained Lipofectamine 2000 as per Figure 22, and the greatly increased competitiveness of magnetofection over short timeframes (as in Figure 28, Figure 29, Figure 31), data was heavily supportive of sedimentation being a major cause of the enhancement of transfection under an oscillating field. This information supports many of the theories proposed in section 5.3.3.1. Furthermore, it suggests that transfection efficiency with magnetofection most likely
cannot improve beyond the best practice of an equivalent transfection agent in non-confluent adherent cells, as once total sedimentation of complexes is achieved, no further circulation of transfection agent can occur, and any complexes which sedimented firmly onto the well surface under magnetic field and failed to contact cells will not subsequently be able to deliver their DNA cargo.

7.4.5.2 Acceleration of Gene Delivery by nTMag+

The current data fails to present much of a case for the improvement of overall transfection efficiency over 4 hour long transfections by utilising magnetofection, as most quantitative measures showed no significant improvement (Figure 27, Figure 28b, Figure 29). There was a better body of data to suggest that magnetofection could be effective at achieving similar outcomes to much longer courses of conventional transfections within a much shorter space of time. Photometry and flow cytometry were ultimately able to observe significant improvements in transfection during timeframes of 1 hour or less.

Enhancement of transfection in this way has relevance to many more difficult targets. Use in vivo, as well as use in vitro with cells that are disrupted, or become unreceptive to gene transfer when exposed to serum free conditions, present considerable challenge to most gene transfer agents. These environments can result in protein corona formation and clearance of nanoparticles in relatively short timeframes even for particles with PEG functionalisation or other moieties designed to prolong operation in serum environments. The prospect of simply accelerating the activity of transfection agents to ensure cell contact and internalisation prior to any of these threats to the transfection being able to take effect is enticing. This concept for gene transfer succeeding in an otherwise hostile, DNA degrading environment through sufficiently rapid delivery has already been utilised as the operating principle behind many respiratory epithelial gene therapy treatments, where a thick mucus layer must be overcome.

It could be assumed that the acceleration in the kinetics of gene delivery seen in experiments is entirely a consequence of relatively rapid sedimentation of transfection complexes towards the bottom of wells, where cells are adhered. This is well supported as discussed earlier in section 7.4.5.1, but there are some issues that should be addressed.

Detracting from this model, uptake of DiI stained Lipofectamine 2000 was as mentioned earlier often not increased or decreased proportionally to transfection efficiency, suggesting
some other mechanism at play. Furthermore, there is some question as to whether the relatively sparse initial plating of cells would logically receive very equal numbers of EGFP transcripts from simple deposition of transfection complexes in a region above the cell, as it would from 4 hours of uptake due to diffusion. Certainly, previous papers have seen substantial improvements in transfection efficiency between magnetofection intervals of 5 minutes and 20 minutes \textsuperscript{42}, which would not be expected in a simple deposition case, despite 5 minutes being close to the time necessary to fully sediment all particles with hydrodynamic diameters less than 1\(\mu\)m to deposit due to magnetic force over small depths such as in well plates as referred to in section 5.3.3.1 \textsuperscript{174}. Lastly, it could be observed in these results that oscillation of magnetic field at a condition relatively similar to optimal, 3Hz x 0.22mm, resulted in less enhancement of transfection efficiency, and less transfection efficiency overall, than others, although given the unusual transfection efficiency observed in Figure 30 this is a less valid concern.

Other previous papers \textsuperscript{42,152–154} have examined oscillating field regimes at various frequencies alongside static magnetic fields and arrived at the conclusion that 2Hz x 0.22mm oscillating magnetic fields offer measurably superior performance to alternatives. If deposition really was the only relevant factor in magnetofection, substantial improvements in deposition over static field could not be expected. While the motion of nanoparticles descending under the effect of the oscillating field could result in better coverage of a cross section near the base of the well plate, due to motion along the x and y axis during descent, modelling in previous publications does not project that this should have a large effect \textsuperscript{174}.

In view of all information on the subject from experimentation with Lipofectamine 2000 / nTMag+, there is reasonable cause to expect that the prospective gene delivery platform candidates explored and rejected during the earlier stages of this research could be more successful and competitive over sufficiently short transfection times, especially with further development of protocols. In particular, while the dendronised polymer did not display highly competitive transfection efficiency, achievement of the observed transfection efficiencies over a sufficiently short time period could constitute a successful reagent, especially in conjunction with features outlined in published work \textsuperscript{56} such as fluorination and other means of additional serum resistance.
7.5 Future Work

This course of research has identified several directions for further study likely to result in novel, potentially effective compounds, or opportunities to better understand magnetofection.

7.5.1 Use of Conjugated SPION Particles as Magnetic Moieties

This project, for association of SPION with existing transfection agents, used electrostatic binding. Electrostatic binding could be expected to be highly reversible, and so there remains a question as to whether superior results could have been achieved with a less reversible addition of SPION. This would also allow for SPION association that does not compromise the electrostatic properties necessary for complexation of transfection agents with DNA, by removing the need to include SPION within the complex. Magnetoliposomes, or more relevant here, SPION connected to cationic dendronised polymer complexes via covalent linkers, would have more reliable association with magnetic moieties in the face of varying pH. Likely schemes would include 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride with N-hydroxysuccinimide (EDC-NHS) reactions previously used in conjugating PEG to citric acid coated SPION. Proper conjugation could potentially allow the use of filtration methods to eliminate loose SPION clusters, leading to more tightly distributed magnetic behaviour among magnetofection complexes.

This would also allow for a more confident understanding of transfection complex contents, which would in turn allow for a more thorough exploration of mechanisms of magnetofection. With the right choice of particle, this would also allow for more confident tracking of the fate of transfection materials within in vivo models using MRI. As it stands, the SPIONs assessed here display characteristics associated with T1 relaxation contrast enhancing agents.

7.5.2 Magnetofection Conditions with Superior Resolution

Challenging or short timeframe conditions for magnetofection do appear in some previous papers, but these results indicate that as a rule, prospective magnetofection agents should be tested as much as possible in harsher conditions that more closely approximate physiological conditions as a standard practice. It is difficult to observe the increased
effectiveness of magnetofection in achieving plasmid expression relative to existing transfection reagents when testing in highly controlled environments with little risk of degradation or clearance of particles, which would rarely be found anywhere in a whole organism. The serum free environments allow gene delivery vehicles with no magnetic moieties or serum resistance to display effective performance in the absence of any real need for haste during transfection.

It could be reasonably expected that in tissue or whole animal models, while the control over magnetic conditions would be diminished, greater exploration of magnetofection’s capacity to improve and direct transfection outcomes could be demonstrated. This would more effectively display the contrast with the low efficacy of conventional transfection in such environments.

It is also worth considering that an important control for future oscillating field magnetofection should be the installation of a non-magnetic dummy plate into the magnet array used. Verifying that there is no mechanical transduction of SPIONs by the motion of magnet arrays themselves could offer potential insights into an overlooked aspect of magnetofection and easily create further understanding of the technique.

With the dendronised polymer examined already possessing prospects for in vivo testing, it would be illuminating to combine its high performing properties with magnetic moieties for an even further improved reagent with spatial targeting and rapid action. Despite the relatively poor performance over long transfection timescales displayed in the flow cytometry data, further development might improve matters. In particular, the 10% to 35% transfection observed when complexed with SPION could be considered good performance if replicated in a much more adverse transfection environment such as full serum, or over an extremely short time.

7.5.3 Cobalt Ferrite Nanoparticles
In addition to the SPION used throughout other stages of the project, oleic acid coated Cobalt Ferrite particles were synthesised as a potential alternative and contrast to more well studied SPION. They were characterised by SQUID as per Figure 32.
Figure 32: Magnetometry data collected from oleic acid coated cobalt ferrite particles following ligand exchange, air dried from hexane. 

(A) The recorded 5K and 300K Loop adjusted to the weight of nanoparticles present, and with the trend from diamagnetic moment removed. (A, Inset) A closer view of the loop intercepts at low field, displaying the lack of measurable coercivity or remanence at 300K.

(B) Zero field cooled (ZFC) and field cooled (FC) curves adjusted to the weight of nanoparticles present.

Cobalt ferrite nanoparticles, as would be expected, are considerably harder magnets than similarly sized SPION, possessing quite significant hysteresis with a remanence of around 50 emu/g and a 20600 Oe coercivity at 5K as can be seen in Figure 32. However, they do not display any coercivity or remanence at 300K, in keeping with the characteristics of a superparamagnetic particle. The particles seem to present a substantially greater blocking
temperature than conventional SPIONs. At the very least, with coating still included, their saturation magnetisation per mass is improved, sitting around 50 – 57 emu/g. In contrast with the SPION examined previously, they are highly superparamagnetic, with no hysteresis observed above the noise floor of the instrument (0.0025 emu).

Cobalt ferrite nanoparticles could be synthesised with a similar level of control to comparable sized SPIONs using simple processes. Substitution of SPION for cobalt ferrite nanoparticles in magnetofection designs could be used to substantially modify magnetofection agents and other SPION based nanotechnology with relative ease. However, there remains a question of whether the properties seen here are actually desirable in nanoparticle applications.

- Any improvements in saturation magnetisation as seen here that can be achieved are generally desirable, as a greater magnetic response can be achieved with less magnetic material.
- While the general blocking temperature of the particles is heightened, it is evidently not enough jeopardise their superparamagnetic behaviour at room temperature.
- Distribution in achievable magnetisation by temperature is slightly more concerning, and may tie in to some unexamined polymorphism within the synthesised particles with particles possessing relatively distinct blocking temperatures from one another.
- It remains likely that any desirable magnetic properties of these particles come at the cost of some increased cytotoxicity, even if effective coating can minimise this.

With these factors considered, it seems unlikely that cobalt ferrite nanoparticles would hold great promise in the context of any of the magnetofection agents assessed here. These cobalt ferrite nanoparticles still behave as relatively soft magnetic materials at physiological temperatures due to superparamagnetic effects, but assuming their hysteresis at 5 °K is indicative of the energy loss possible per particle, they should perform well in excess of any SPION in roles requiring heat generation by oscillating magnetic field. They appear to possess some superior properties as a soft magnetic nanoparticle, even if a roughly 20% increase in saturation magnetisation alone does not seem significant enough to compensate for the substantially elevated risk of toxicity that comes with cobalt use.

Nonetheless, the use of cobalt ferrite nanoparticles as part of a novel magnetofection agent might hold unexplored benefits relative to conventional SPIONs. It seems likely that cobalt ferrites with these characteristics could achieve superior MRI contrast to SPIONs, which
would have relevance \textit{in vivo}. Thermally labile linker based DNA delivery might also be an appropriate application for particles with these observed properties \cite{198}. Additionally, with the role of magnetofection other than sedimentation unknown, cobalt ferrites may be able to assist endosomal escape through relatively minor increases in heat generation or cytotoxicity, which have an established role in DNA uptake behaviours \cite{5}.

7.5.4 The Effect of Oscillating Magnetic Fields and SPION on Endocytotic Pathways

The mechanism of uptake of transfection complexes during oscillating field magnetofection has been examined before \cite{158}. However, previous studies have only established that conventional endocytosis pathways are responsible for uptake. Evidence exists that the motion of SPION conjugated directly to mechanosensitive proteins present in cell membranes can facilitate accelerated rates of endocytosis \cite{179,180}. Even though the DiI signal in this case did not seem to support this as a contributing factor to oscillating field magnetofection, DiI can only account for the fate of lipoplexes, which may be influenced by other factors.

Therefore, to provide superior insight into the mechanism of magnetofection, the rate of endocytosis of cells during transfection could be more monitored in some fashion, perhaps by uptake of diffuse and suspended non-membrane binding particles unlikely to be exocytosed, while typical magnetofection complexes used as treatment undergo exposure to an oscillating magnetic field. Identifying a mechanism to magnetofection beyond sedimentation, if one exists, would have flow on effects to all drug or gene delivery particles that need to contend with facilitated cellular entry.
7.6 Conclusions

This research work has been able to reach several key conclusions.

- Magnetofection utilising nTMag+ can achieve maximum transfection for Lipofectamine 2000 over a shorter transfection time, on the order of 1 hour or less, but cannot reliably increase the maximum transfection efficiency achievable under ideal conditions. It is likely that this is true to some extent for other magnetofection agents, possibly to a greater extent than has been implied by literature.

- The deposition of transfection complexes onto cells is the most significant mechanism behind the improvements to transfection offered by magnetofection. However, it is also likely that it is not responsible for the entirety of enhanced transfection efficiency of transfection complexes containing magnetic particles in the presence of an oscillating magnetic field.

- Other prospective magnetofection agents examined besides nTMag+, particularly the dendronised co-polymer, may have been capable of much more competitive performance relative to Lipofectamine 2000 than was observed. This would require further structural and procedural optimisation, but especially transfections conducted over much shorter timeframes, and in challenging conditions with serum or DNase present.

Gene therapy is an important developing therapeutic for conditions with enormous relevance in medicine. High performing non-viral treatments present a more reliable and safe means of achieving effective gene expression in vivo. Spatial targeting enabled by magnetofection greatly increases the suitability of speculative non-viral gene transfection agents for future clinical treatment of tumours. The insights gained in this study regarding ideal development models for future magnetofection agents should ideally inform more efficient identification of novel and high performing vehicles compatible with magnetofection in future.
8 References


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