Magnetic nanoparticle assisted gene delivery

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TECHNOLOGICAL
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MAGNETIC NANOPARTICLE
ASSISTED GENE DELIVERY

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2012

**MAGNETIC NANOPARTICLE
ASSISTED GENE DELIVERY**

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ABSTRACT

Gene delivery is the process of delivering foreign DNA into host cells. Viruses are commonly used because of their very high transfection efficiency. Unfortunately, viral based delivery systems have serious safety concerns such as insertional mutagenesis and severe immune response. In contrast, non-viral based delivery systems such as magnetically enhanced gene delivery technique have good safety profile.

In the magnetically enhanced gene delivery technique, DNA complexed with polymer coated aggregated magnetic nanoparticles (AMNPs) is used for effecting transfection. The main objective of this project is the synthesis, characterization, property evaluation and performance of MNPs in *in vitro* studies of gene delivery.

Our hypotheses are as follows: PEI-AMNPs will yield higher transfection efficiency than PEI due to the higher concentration of complexes on the cell surface. PEI-AMNPs with better magnetic properties (higher saturation magnetization and susceptibility), lower N/P ratio and smaller complex size will result in higher transfection efficiency than PEI-AMNPs with poorer magnetic properties (lower saturation magnetization and susceptibility), higher N/P ratio and larger complex size. The mechanism of magnetic transfection is endocytosis.

In vitro studies of transfection efficiency in COS-7 cells were carried out using reporter genes (pEGFP-N1 and pMIR-REPORT) complexed polyethylenimine (PEI)

coated iron oxide magnetic nanoparticles. PEI coated AMNPs (PEI-AMNPs) with average individual particle diameters in the range of 8 nm to 30 nm were studied and characterized by transmission electron microscopy, vibrating sample magnetometry, X-ray diffractometry, thermal gravimetric analysis and photon correlation spectroscopy methods.

For MNP performance, PEI coated aggregates of magnetic nanoparticles of 8 nm and 30 nm in diameter (PEI-A8MNP and PEI-A30MNP respectively) yielded higher transfection efficiency compared to commercial polyMAG particles as well as PEI of equivalent molar ratio of nitrogen/phosphorous (N/P ratio). The transfection efficiency was related to the physical characteristics of the PEI-AMNPs and its complexes: transfection efficiency was strongly positively correlated with saturation magnetization (M_s) and susceptibility (χ), strongly negatively correlated with N/P ratio and moderately negatively correlated to hydrodynamic diameter of the complex. PEI-A8MNP and PEI-A30MNP possessing higher M_s , χ , lower N/P ratio and smaller complex size exhibited higher transfection efficiency compared to PEI-A16MNP which has weaker magnetic properties, higher N/P ratio and larger complex size.

The effects of static and pulsed magnetic fields on the transfection efficiency and viability were also studied. An optimum magnetic field of 130 mT yields maximum transfection efficiency with good viability. Pulsed magnetic fields did not enhance transfection efficiency. From these studies, it was demonstrated that optimization of the physical properties of PEI-AMNPs and the external magnetic field is necessary to maximize transfection efficiency.

Normal, reverse, retention magnetic transfection experiments, cell wounding assays and confocal laser scanning microscopy (CLSM) were performed to gain insights into the mechanism of magnetic transfection. The results of the normal, reverse and retention magnetic transfection experiments showed that highest transfection efficiency was achieved in the normal magnetic transfection mode due to a clustering of PEI-AMNPs to the cells. Cell wounding assays and CLSM results suggested that the mechanism of magnetic transfection is through endocytosis rather than cell wounding.

The two major contributions of this research project are: (1) Physical parameters such as magnetic properties, N/P ratio, size of the complex and the applied magnetic field strength significantly affect the amount of complex taken into the cell; these parameters need to be optimized in order to obtain high transfection efficiency with reasonable cell viability. It was validated that PEI-AMNPs with better magnetic properties (higher saturation magnetization and susceptibility), lower N/P ratio and smaller complex size will result in higher transfection efficiency than PEI-AMNPs with poorer magnetic properties (lower saturation magnetization and susceptibility), higher N/P ratio and larger complex size. (2) In magnetic nanoparticle assisted gene delivery, magnetic complexes clustered onto the cell surface quickly upon application of an external magnetic field; they entered the cell via endocytosis and not by cell wounding.

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CHAPTER 1: INTRODUCTION

This chapter presents an overview of nanotechnology applications in drug delivery, followed by the motivation, objectives, significance and novelty of this project. The scope, hypothesis, methodology and significant findings from this work are also described.

1.1 Nanotechnology: Overview of applications in drug delivery

Nanotechnology can be defined as the science and engineering involved in the design, synthesis, characterization and application of materials and devices with at least one functional dimension on the nanometer scale. One nanometer is one-millionth of a millimetre, a single human hair is around 80,000 nanometers in width. Nanotechnology is not a single scientific discipline but an union of established sciences such as chemistry, physics, biology and materials science [1].

The specific application of nanotechnology for the prevention, treatment and cure of diseases is called nanomedicine [2-4]. The majority of work in nanomedicine can be classified in one of the following three areas: (1) therapeutic delivery systems with the potential to deliver genes and pharmaceuticals; (2) biosensors, biochips, and novel imaging techniques for diagnostic monitoring and imaging; (3) novel biomaterials and tissue engineering for active tissue regeneration. The use of nanotechnology in medicine is a rapidly expanding field, especially in the areas of drug delivery and *in*

vivo imaging [5-8]. It was reported that 95% of all new potential therapeutics have poor pharmacokinetics and biopharmaceutical properties [9]. Thus, it is critical to develop new drug delivery systems that are able to distribute the therapeutic drugs to the site of interest without damaging healthy organs and tissues. Table 1.1 shows the nanoscale systems for drug delivery using liposomes, micelles and nanoparticulate systems. Table 1.2 shows nanoscale systems for imaging.

Table 1.1: Nanoscale systems for drug delivery [5].

Drug delivery systems	Stage of development	Limitations of use	Examples of application
Liposomes	Marketed	Preparation steps have to be carefully controlled to achieve reproducible properties such as size and entrapment efficiency	Amphotericin B Daunorubicin Doxorubicin
Micelles			
Phospholipid	Preclinical	Limited stability in aqueous medium compared to other micelle types	Paclitaxel Camptothecin Diazepam
Pluronic®	Clinical Preclinical	Some monomers have not been tested in humans	Doxorubicin (SP1049C) Paclitaxel Tamoxifen Etoposide
Poly (L-aminoacid)	Clinical	Immune response may increase with diversity in amino acids used. Biodegradability of poly(amino acids) requires validation	Doxorubicin (NK911) Antisense oligonucleotides
Polyester	Preclinical	Polyester degrades by hydrolysis to produce acid metabolites that in excess may not be desirable	Paclitaxel Doxorubicin
Nanoemulsions	Preclinical	High surfactant concentration of 20% and higher may be required in the formulation	Amphotericin B Paclitaxel Dexamethasone Benzathine penicillin G
Nanoparticulate systems			
Drug nanocrystals	Preclinical	Polymers and surfactants covering nanocrystal surfaces are required to provide stabilization against	Amphotericin B Etoposide Camptothecin Paclitaxel

		aggregation	
Polymer-based nanoparticles	Preclinical	Polyester degrades by hydrolysis to produce acid metabolites that in excess may not be desirable	Tamoxifen Cyclosporin-A Theophylline
Lipid-based nanoparticles	Preclinical	Enzymatic degradation in vivo can lead to production of undesirable metabolites such as stearic acid	Doxorubicin Camptothecin
Ceramic-based nanoparticles	In vitro	Release of encapsulated drugs may be problematic	2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide
Albumin nanoparticles	Marketed In vitro	Validation of lack of immune reactivity maybe required	Paclitaxel DNA and antisense oligonucleotides
Nanogels	Preclinical	Some polymers used may not yet be tested/used in humans	Oligonucleotides
Dendrimers	Preclinical In vitro	Positive charge on dendrimer surface may lead to toxicity and immunogenicity	Indometacin 5-fluorouracil Antisense oligonucleotides

Table 1.2: Nanoscale systems for imaging [5].

Drug delivery systems	Stage of development	Technique	Contrast agent/imaging agent/radiolabel	Limitations of use
Liposomes	Preclinical	SPECT MRI PET	^{99m} Tc Gadolinium ([2-18F]FDG)	Preparation steps have to be carefully controlled to achieve reproducible properties such as size and entrapment efficiency
Quantum dots/nanocrystals	Preclinical	Optical/fluorescence	Quantum dots Quantum dots-micelles Quantum dot-conjugates	Further safety studies are required because quantum dots are very stable
Magnetic nanoparticles	Clinical Preclinical Cellular	MRI	Iron oxide-dextran Iron oxide-polyacrylamide Iron oxide-SLN Iron oxide-insulin	Toxicity may occur due to cellular internalization and membrane disruption
Dendrimers	Preclinical	MRI	Gadolinium	Positive charge on

				dendrimer surface may lead to toxicity and immunogenicity
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MRI = magnetic resonance imaging; PET = positron emission tomography; SLN = solid lipid nanoparticles; SPECT = single photon emission computed tomography.

The enormous interest in nanomaterials is due to the discovery of their unusual physical and chemical properties. A key reason for the change in the physical and chemical properties of small particles as their size decreases is the increased fraction of surface atoms, which experience an environment (coordination number, symmetry of the local environment, etc.) differing from those of the bulk atoms. From an energy standpoint, a decrease in particle size results in an increase in the surface energy contribution [10].

Some nanomaterials used in biomedical applications are [6, 8, 11-14]:

- Fullerenes, C₆₀, (0.7 nm): a functional drug carrier with linked antibodies or other targeting agents on the surface carbon atoms.
- Dendrimers (5-50 nm): the branched structure allows us to link labels, probes and drugs for drug carrier, implanted sensor and medical device applications.
- Nanoparticles (< 100 nm): inorganic or organic, in implanted materials, as nanoshells and nanoemulsions for drug delivery; quantum dots (< 8 nm) and magnetic nanoparticles for labeling in diagnostics and implanted sensors and medical devices.
- Carbon nanotubes (1 nm for single wall and 10-100 nm for multiwall): one dimensional fullerene nanoelectrode arrays for *in vitro*, *in vivo* and implanted sensors and medical devices.

- Single crystal nanowires (5-100 nm): one-dimensional nanosystems, capable of applications similar to those of nanoparticles and carbon nanotubes.

Magnetic nanoparticles offer some attractive possibilities in nanomedicine [15-26]:

(i) Figure 1.1 shows the types of nanoparticles used in drug delivery and their sizes relative to biological entities. The size of magnetic particles can be controlled in the nanometer range. At these nanometer dimensions they are smaller than or comparable to those of a cell (10 – 100 μm), virus (20 – 450 nm), protein (5 – 50 nm) or gene (2 nm wide and 10 – 100 nm long). The nanoparticles can be coated with biological molecules to enable them to bind to a biological entity, thereby giving us a controllable means of ‘labelling’ the entity.

Figure 1.1. Nanoparticle systems for drug delivery applications [27].

(ii) An external magnetic field gradient is used to control these magnetic nanoparticles. The ability of the magnetic fields to penetrate into human tissue opens

up useful applications such as delivering of magnetically tagged anticancer drugs or radionuclides to a targeted region of the body, such as a tumour. In the case of gene therapy, therapeutic genes can be delivered.

(iii) If an alternating magnetic field is used, energy from the magnetic field can be transfer to the magnetic nanoparticles. The ability to heat these particles enables their use as hyperthermia agents, which delivers lethal amount of thermal energy to targeted bodies such as tumours. They can also be used to enhance chemotherapy and radiotherapy where tissue warming results in more effective malignant cell destruction. While iron oxide nanoparticles are more widely used, magnetic nanoparticles with higher dipole moment such as FePt and FeCo have been proposed because they generate more heat per unit mass of particles [28, 29].

The magnetic material commonly used in gene delivery is superparamagnetic iron oxide [17, 30-36]. Particles smaller than the size of a single magnetic domain can exhibit superparamagnetic behaviour, i.e., they do not retain magnetization when an external magnetic field is removed [37]. Hence, aggregation of these particles is reduced. While this may be useful, they also tend to have smaller moments. Aggregation can also be prevented by steric hindrance of aggregation by appropriate choice of surfactants and polymeric coatings. The nucleic acid which the magnetic nanoparticles delivers is a polyanion consisting of phosphate group-repeated chains. Since the membrane of the target cells is also negatively charged, the nucleic acid cannot easily interact with the cell due to electrostatic repulsion, hence a coating material is required. Polyethylenimine (PEI) is the preferred choice of coating material around the magnetic particles, it is an excellent transfection vector due to the

“proton sponge” effect, which causes endosomal escape and hence avoids lysosomal degradation of nucleic acids [38-41].

1.2 The motivation: Gene therapy

Gene therapy represents an exciting development in medical treatment. It promises to revolutionize medicine as it aims to treat or eliminate the causes of diseases rather than the symptoms [42]. Gene therapy has an important potential advantage of being able to treat a wide range of diseases such as cancer, inherited monogenic disorders, infectious diseases, cardiovascular diseases, rheumatoid arthritis and cubital tunnel syndrome [43].

There are two categories of gene delivery systems, namely viral vectors and non-viral vectors. There are diverse materials that can be used for non-viral delivery of genes, such as polymers, lipids, carbon nanotubes and engineered peptides [44]. Compared to viral vectors, non-viral delivery systems offer several advantages such as ease of production, reduced cytotoxicity and immunogenicity. However, their use has been limited by the relatively low transfection efficiency [43]. Thus, the majority of clinical studies are based on viral vectors. However, a big disadvantage of using viral vectors is the safety concerns due to the generation of replication-competent virus during manufacturing or after administration to patients [45]. Table 1.3 shows the advantages and disadvantages of viral and non-viral gene transfer vectors.

Table 1.3. Advantages and disadvantages of gene-transfer vectors [43, 45].

Vector	Advantages	Disadvantages
Viral		
Adenovirus	Very high transfection efficiency ex vivo and in vivo. Transfects proliferating and non-proliferating cells. Substantial clinical experience acquired. Efficient retargeted transfection demonstrated.	Repeat dosing ineffective owing to strong immune responses. Insert-size limit of 7.5 kb. Manufacture, storage, quality control are moderately difficult. Short duration of expression.
Retrovirus	Fairly prolonged expression. High transfection efficiency ex vivo. Substantial clinical experience ex vivo. Low immunogenicity.	Low transfection efficiency in vivo. Insert-size limit of 8 kb. Transfects only proliferating cells. Safety concern of insertional mutagenesis. Manufacture, storage, quality control are extremely difficult.
Lentivirus	Transfects proliferating and non-proliferating cells. Transfects haematopoietic stem cells.	Safety concerns from immunodeficiency virus origins. Manufacturing, storage, quality control are extremely difficult. Insert-size limit of 8 kb. No clinical experience.
Adeno-associated virus	Efficiently transfects a wide variety of cells in vivo. Very prolonged expression in vivo. Low immunogenicity.	Insert-size limit of 4.5 kb. Manufacture, quality control are very difficult. Little clinical experience. Safety concern of insertional mutagenesis. Repeat dosing affected by neutralizing antibody responses.
Non-viral		
Naked DNA	Manufacturing, storage, quality control are simple and cheap. Very low immunogenicity. Clinical efficacy demonstrated in critical limb ischaemia. Very good safety profile.	Very short duration of expression in most tissues. Very inefficient transfection ex vivo and in vivo. Retargeting transfection very difficult.
Condensed DNA particles	Relatively simple manufacturing, storage, quality control. Efficient transfection ex vivo. Low immunogenicity. Good safety profile. Retargeted transfection	Inefficient transfection in vivo. Very short duration of expression. No clinical experience.

	demonstrated.	
Cationic lipids	Relatively simple manufacturing, storage, quality control. Efficient transfection ex vivo. Low immunogenicity. Good safety profile.	Inefficient transfection in vivo. Very short duration of expression. Little clinical experience. Retargeting transfection difficult.
Liposomes	Completely synthetic. No limitation on size and type of nucleic acid.	No targeting. Inefficient.
Mechanical administration	No limitation on size of nucleic acid.	No targeting. Inefficient. Possible requirement for surgical procedure.

In 2006, an international group of scientists reported success in using gene therapy to effectively treat two adult patients for a disease affecting nonlymphocytic white blood cells called myeloid cells [46]. Myeloid disorders are common and include a variety of bone marrow failure syndromes, such as acute myeloid leukemia. Retroviral vectors were used to deliver the gene encoding the missing protein into the genome. This study was the first to show that gene therapy can cure diseases of the myeloid system.

In 2006, another group of researchers successfully reengineered immune cells, called lymphocytes, to treat seventeen patients with advanced metastatic melanoma [47]. Retrovirus was used to carry genes encoding specific proteins (T cell receptors (TCRs)) into the cells. This was the first time that gene therapy was used to successfully treat cancer in humans.

In 2008, the first clinical trial to use gene therapy to treat a type of inherited blindness was performed [48]. It involved three young patients with a condition called Leber's

congenital amaurosis (LCA). Adeno-associated virus was used. The trial was a success, sight was improved and the treatment is safe.

1.3 The problem: major setbacks of viral gene delivery

Although a few successful human gene therapies were reported in recent years, the Food and Drug Administration (FDA) has not yet approved any human gene therapy product for sale. This is mainly due to three major setbacks since the first gene therapy clinical trial began 20 years ago.

In 1999, 18-year-old Jesse Gelsinger died from multiple organ failures during a gene therapy trial for ornithine transcarboxylase deficiency (OTCD). A severe immune response to the adenovirus carrier is believed to be the cause of his death [49].

In 2002, ten children had been successfully treated by gene therapy for X-linked severe combined immunodeficiency disease (X-SCID), also known as "bubble baby syndrome". However, two of them developed a leukemia-like condition [50]. This was traced to the retrovirus vector integration near the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2 [51].

In the latest setback to viral gene delivery, a human volunteer, Jolee Mohr died in 2007, three weeks after receiving a second injection of an adeno-associated virus vector in a gene therapy trial for arthritis [1].

1.4 The solution: non-viral gene delivery systems

As to diseases, make a habit of two things – to help, or at least to do no harm. --- Hippocrates, 460-377 BC.

Serious adverse consequences resulting from the use of viral delivery systems serve as an impetus to improve non-viral gene delivery systems which are potentially safer. However, the delivery of nucleic acids by non-viral methods such as *Lipofectamine* (a commonly used cationic liposome based reagent for *in vitro* transfection [52]) is a diffusion limited process, which can result in only a small percentage of nucleic acids reaching target cells. This limitation is even more pronounced *in vivo* since the target tissue is often a highly viscous material [53, 54]. Another major drawback of cationic liposomes is its toxicity in *in vivo* gene delivery [55].

Earlier work has shown that by applying an external magnetic field to magnetic nanoparticles (MNPs) complexed with nucleic acids, efficient delivery of nucleic acids to the target cells can be effected [56]. Such magnetic particle assisted gene delivery, also known as magnetic transfection, is a recently developed physical non-viral delivery system [17]. Efficient gene delivery occurs under the influence of a magnetic field acting on the nucleic acid/magnetic particle complexes, resulting in rapid and substantial complex-target cell contact (Figure 1.2).

Figure 1.2. Principle of magnetic transfection: (1) Polyelectrolyte-coated aggregated magnetic nanoparticles (AMNPs) are mixed with DNA. (2) These DNA/polyelectrolyte-coated AMNPs complexes are added to cells in the culture dish. (3) The cell culture dish is placed on top of a magnet for 5 to 30 min of incubation. The magnetic field rapidly pulls the complexes onto the cells to be transfected, resulting in rapid kinetics and efficient gene delivery as most of the complexes get in contact with the cells. (4) The DNA/polyelectrolyte-coated AMNPs complex enters the cell nucleus via endocytosis. (5) If plasmids coding for green fluorescence protein are used, the cell becomes green, indicating successful transfection.

Magnetic transfection has been successfully tested *in vitro* [17, 30, 32, 33, 57-64] and *in vivo* [17, 32, 33, 53, 60, 65-67] on several immortalized cell lines (e.g. CHO, COS-7, HeLa, HEK-293, NIH-3T3) as well as primary cells (e.g. HUVECs, neurons, aortic endothelial cells, nasal airway epithelium) [68]. Depending on the cell type, *in vitro* studies showed that cells incubated in a magnetic field displayed gene expression up to several hundred-fold greater than those not exposed to the magnetic field, the time required was reduced from ~4 h typically used in standard transfection techniques to 5 min for magnetic transfection [53, 69]. Magnetic transfection also reduces the amount of nucleic acids required to effect similar level of transfection compared to standard vectors such as Metafectene [53] and is extremely useful for gene delivery of difficult-to-transfect or otherwise non-permissive cells such as tumor cells [59, 60].

1.5 Objectives, significance and novelty

The main objective of this project is the synthesis, characterization, property evaluation and performance evaluation of DNA complexed PEI coated iron oxide MNPs for *in vitro* studies of gene delivery. A recent search of the National Center for Biotechnology Information's (NCBI) MEDLINE database [70] for the keywords 'magnetic transfection' yielded less than 1000 papers out of more than 140,000 papers on transfection. While much research has been done on conventional transfection involving viral or non-viral techniques, little information is available on the process of magnetic transfection.

Magnetic transfection is a technique which offers the potential of significantly increased transfection efficiency [17], however, aspects related to the magnetic carriers have not been well studied. The significance of this research lies in the studies of the relationship between transfection efficiency and physical parameters such as magnetic properties, particle size, hydrodynamic diameter, zeta potential and N/P ratio of the magnetic carriers, magnetic field strength/magnetic field gradient and type of magnetic field. Using this knowledge, MNPs can be developed to achieve optimum transfection efficiency and cell viability.

The novelty of this work lies in the use of modified forms of magnetic transfection, i.e., reverse and retention magnetic transfections, confocal laser scanning microscopy and cell wounding assays to study the mechanism of magnetic transfection. These studies are detailed under Methodology in section 1.7.

In short, the objectives are to determine which physical parameters play a significant role in affecting transfection efficiency in magnetic transfection and also to determine is cell wounding a possible operating mechanism.

1.6 Scope

The overview of this thesis is outlined in Figure 1.3. For materials selection, the MNPs used for magnetic transfection are commonly superparamagnetic iron oxides [30, 31, 58, 71, 72]. Particles smaller than the size of a single magnetic domain can exhibit superparamagnetic behaviour, i.e., they do not retain magnetization when an external magnetic field is removed [10]. Hence, aggregation of these particles is reduced. Iron oxides are also biocompatible and are clinically used as contrast agents in magnetic resonance imaging [73]. PEI was used as the polymer coating as it is an excellent transfection vector [38-41].

Most magnetic transfection studies explored the feasibility of using this technique for various cell types, very few studies [34, 74, 75] focused on the physical characteristics of the magnetic particles needed for high transfection efficiency. Surprisingly, there are no studies on the effect of magnetic properties of the MNPs on transfection efficiency, although this information is important for magnetic transfection to work effectively. Optimizing the magnetic properties would enhance transfection efficiency and could also potentially affect the mechanism of magnetic transfection. Hence, synthesis, characterization, property and performance evaluation of the coated AMNPs was performed and their correlation with transfection efficiency was examined. In this part of the work, the variables are the magnetic properties and N/P ratio of the coated AMNPs and hydrodynamic diameter of the complex. The constants are the cell type

(COS-7 cells), coating material (branched 25 kDa PEI), magnetic field strength (130 mT) and incubation time (20 min).

The existing literature has demonstrated the advantages of magnetic field on transfection efficiency, however no study has been conducted to examine the dependence of transfection efficiency on magnetic field strength. Recently, researchers have used pulsed magnetic field (maximum amplitude of 27 mT) to enhance magnetic transfection [30, 62, 76, 77]. Transfection level was increased up to ten-fold compared to static fields. Therefore, we studied the effect of different static magnetic field strength and pulse frequencies on transfection efficiency and cell viability in COS-7 cells. In the static magnetic field strength study (0 – 250 mT), the properties of the particles and incubation time were fixed. For the pulsed magnetic field study, one set of particles was subjected to various frequencies (0 – 500 Hz) under constant magnetic field strength and incubation time. We also seek to understand whether the magnetic complexes enter the cell via cell wounding due to the higher magnetic field strength and pulse frequency.

1.7 Hypothesis

PEI-AMNPs will yield higher transfection efficiency than PEI due to the higher concentration of complexes on the cell surface. PEI-AMNPs with better magnetic properties (higher saturation magnetization and susceptibility), lower N/P ratio and smaller complex size will result in higher transfection efficiency than PEI-AMNPs with poorer magnetic properties (lower saturation magnetization and susceptibility), higher N/P ratio and larger complex size. The mechanism of magnetic transfection is endocytosis.

Figure 1.3. Flow diagram showing the overall approach used in this project. Suitable materials were selected, synthesized, characterized and optimized. The effects of physical parameters, static and pulsed magnetic fields on transfection efficiency were studied. The mechanism of magnetic transfection was also examined.

1.8 Methodology

Iron oxide magnetic nanoparticles (MNPs) of average particle diameter of 8 nm, 16 nm and 30 nm diameter were synthesized via modifications of the co-precipitation technique. These MNPs were subsequently coated with 25 kDa branched PEI (Figure 1.4).

Figure 1.4. Schematic of (A) MNPs, (B) PEI-coated AMNPs and (C) plasmid complexed PEI-coated AMNPs. Positively charged PEI molecules attached to the negatively charged MNPs by physisorption (van der Waals interaction). The negatively charged plasmid complexed with the positively charged PEI-AMNP via electrostatic interaction. Variables that affect transfection efficiency include magnetic properties, N/P ratio, zeta potential, hydrodynamic diameter, magnetic field strength and type of magnetic field.

The particle morphology, size and distribution were characterized using transmission electron microscopy (TEM), the phase was determined by X-ray diffractometry (XRD) and the hydrodynamic diameter and surface charge using photon correlation spectroscopy (PCS). The magnetic properties were measured by vibrating sample magnetometry (VSM). The PEI content on the AMNPs was determined by thermogravimetric analyzer (TGA).

In vitro transfection and viability studies on COS-7 cells were performed using pEGFP-N1 and pMIR-REPORT complexed PEI-coated AMNPs (Figure 1.4C). Plasmid pEGFP-N1 codes for green fluorescence protein (GFP) under the control of cytomegalovirus (CMV) promoter. Plasmid pMIR-REPORT contains a firefly luciferase reporter gene under the control of a CMV promoter. Quantification of transfection efficiency and cell viability was performed using luciferase assay and crystal violet staining assay, respectively. The correlation between the transfection efficiency and physical parameter was determined by regression analysis.

A study on the effect of static magnetic field strength was performed using a permanent magnet placed at various distances from the cells. A study of pulsed magnetic field with different frequencies also was performed using an electromagnet powered by a pulse power supply.

To understand the mechanism of magnetic transfection, modified forms of magnetic transfection were performed, i.e., reverse magnetic transfection and retention magnetic transfection (Figure 1.5). It was hypothesized that if endocytosis occurs, the transfection efficiency and viability of reverse magnetic transfection would be similar to that of normal magnetic transfection. Also, the transfection efficiency of retention magnetic transfection would be lower than that of reverse magnetic transfection since the magnetic field would have reduced the chances of DNA/PEI-AMNP complexes being taken into the cell via endocytosis.

Figure 1.5. Schematic of (A) normal, (B) reverse and (C) retention magnetic transfection. In normal magnetic transfection, cells were seeded one day before transfection. Transfection was performed with DNA/PEI-AMNP complexes under 20 min of magnetic field application. The magnetic field was removed and incubation was continued for 48 h. In reverse and retention magnetic transfection, DNA/PEI-AMNP complexes were added and settled under 20 min of magnetic field application before adding the cells. In retention magnetic transfection, the magnetic field was applied until luciferase and viability assays were conducted 48 h after transfection.

Modeling was also carried out to estimate the time taken for a MNP to enter the cell. Cell wounding assays to determine plasma membrane disruption during magnetic transfection and confocal laser scanning microscopy (CLSM) for intracellular tracking were performed.

All biological data are expressed as mean $\pm$ standard deviation. Luciferase activity, transfection efficiency and viability were analyzed by the single-factor ANOVA test. A p-value (p) < 0.05 was considered significant.

1.9 Significant findings

- a. Iron oxide MNPs of average individual particle diameter of 8 nm, 16 nm and 30 nm were synthesized by the co-precipitation technique. XRD analysis showed that 8 nm and 30 nm MNPs were magnetite and 16 nm MNPs were maghemite. This explains why the saturation magnetization of 16 nm MNPs is lower than that of 8 nm MNPs.
- b. 8 nm and 16 nm MNPs exhibit superparamagnetic behaviour, whereas 30 nm does not. The saturation magnetization values of coated MNPs were only slightly lower than those of the uncoated MNPs, demonstrating that the PEI coating did not significantly affect the magnetic properties. The saturation magnetization values for coated 8 nm, 16 nm and 30 nm MNPs were 41, 27 and 75 emu/g, respectively.
- c. Transfection efficiency was increased ~32 times by the use of MNPs compared to PEI with equivalent N/P ratio. Transfection efficiency of MNPs under a magnetic field was four to seven times higher than that of MNPs not subjected to a magnetic field. The use of MNPs dramatically reduced incubation time from 5 h to 20 min and considerably decreased the amount of PEI required to achieve similar level of transfection. PEI with a N/P ratio of 7.5 was needed to achieve transfection efficiency similar to that of MNPs coated with PEI having N/P ratio equal to 2.2. The significant increase in transfection efficiency, much shorter incubation time and smaller amount of PEI required illustrate the advantages of magnetic transfection.

-
- d. Transfection efficiency was strongly positively correlated with magnetic properties (M_s and χ), strongly negatively correlated with N/P ratio and moderately negatively correlated to hydrodynamic diameter of the complex. MNPs with higher transfection efficiency have higher M_s and χ , lower N/P ratio and smaller complex size. MNPs with inferior magnetic properties require longer incubation time to achieve the same level of transfection as MNPs with superior magnetic properties.
- e. An optimum magnetic field exists for maximum transfection. For PEI-coated 8 nm and 30 nm AMNPs, the magnetic field required for maximum transfection efficiency is 130 mT with incubation time of 20 min. For these conditions, their transfection efficiencies were ~45% higher than polyMAG, a commercial magnetic formulation.
- f. Pulsed magnetic field of 600 mT with frequency ranging from 0 to 500 Hz has no effect on transfection efficiency and viability, contrary to previous reports. This could indicate the importance of the horizontal component of the pulsed magnetic field.
- g. Higher concentration of complexes per cell resulted in higher transfection efficiency and lower viability.
- h. Mechanism studies using modified forms of magnetic transfection, CLSM, cell wounding assay and modeling suggest that the mechanism of magnetic transfection is via endocytosis rather than cell wounding.
-

1.10 Organization of thesis

This thesis is divided into six chapters:

Chapter 1 presents the motivation for using gene therapy to treat diseases, problems associated with viral gene delivery and solution using magnetic transfection provides the objective for this thesis. The scope, significance, novelty, methodology and a summary of the significant results are also included.

Chapter 2 comprises an overview of viral and non-viral gene delivery methods, barriers to non-viral gene delivery, magnetic vectors for magnetic transfection, as well as a literature review of magnetic transfection.

Chapter 3 lists experimental procedures and characterization techniques carried out in this study. The procedures include synthesis, characterization and property evaluation of DNA complexed PEI-coated iron oxide MNPs, *in vitro* transfection, luciferase and viability assays, normal, reverse and retention magnetic transfection, confocal laser scanning microscopy, and cell wounding assays.

Chapter 4 contains the results obtained from characterization, optimization studies, magnetic field studies and mechanism studies.

Chapter 5 is a discussion of MNP properties, correlation between transfection efficiency and physical parameters, the effect of different magnetic field strength and frequencies, and mechanism studies.

Chapter 6 summarizes the conclusions of this project.

The next chapter covers an overview of selected topics on viral and non-viral gene delivery methods, barriers to non-viral gene delivery, magnetic vectors for magnetic transfection, as well as a literature review of magnetic transfection.

CHAPTER 2: BACKGROUND

In this chapter, different types of gene delivery techniques, mechanism and barriers to non-viral gene delivery are presented. The past developments and factors affecting magnetic transfection are summarized. The magnetic behaviour of these magnetic nanoparticles, their synthesis and coating materials are also described.

2.1 Gene therapy and gene delivery techniques

Genes, which are carried on chromosomes, are the basic physical and functional units of heredity [78]. Genes are specific sequences of bases that encode instructions on how to make proteins, which perform most of life functions. Genetic disorders happen when genes are altered and produce encoded proteins that are unable to carry out their normal functions.

Gene therapy represents an exciting development in medical treatment because it can correct the defective genes responsible for disease development [42]. In such a treatment, genetic disorders are rectified by the insertion of corrective genetic material into target cells to produce therapeutic agents *in situ* to stimulate the immune system. Therefore, gene therapy aims to cure diseases rather than just treating the symptoms of diseases [79]. The techniques of introducing foreign genetic material into cells are classified into two categories; virus-based and nonviral methods.

2.1.1 Viral gene delivery techniques

Viruses are the most common vectors used because of their high transfection efficiency. This is understandable as viruses have evolved, through millions of years, a method of encapsulating and delivering their genes to human cells in a pathogenic manner. Hence, scientists have tried to take advantage of this capability and manipulate the virus genome by removing the disease-causing genes and inserting therapeutic genes. This was made possible in the early 1980s with the invention of recombinant DNA technology in 1973 [80]. Many types of viruses have been adapted as vectors [42, 81, 82]. The most advanced are adenovirus (Ad), retrovirus (Rv) and adeno-associated virus (AAV) [43].

2.1.1.1 Adenovirus (Ad) vectors [43, 45, 83, 84]

Ad is a class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans [85, 86] (Figure 2.1). The virus that causes the common cold is an adenovirus. Ad vectors can transfect a wide variety of cell types and both proliferating and quiescent cells with high transfection efficiency. Ad vectors do not integrate into the chromosome of the target cell and hence the gene expression is transient and usually become undetectable after two weeks. Ad vectors have an insert-size limit of ~7.5 kb. A serious disadvantage of Ad vectors is their tendency to cause strong immune response because the gene is delivered with many residual viral genes [87, 88]. At high doses, neutralizing antibodies are directed to the proteins of the virus which prevents it from binding to target cells. Subsequent dosing becomes ineffective.

Figure 2.1. Schematic of adenovirus [89].

Among the viral vectors, Ad is the easiest to manufacture. However, Ad, as with other viral vectors, has a contamination problem of replication-competent virus (RCV) in the preparation. RCV arises from recombination between viral sequences in the vector and in the chromosome of the producer cells. Its presence limits the maximum administration dose of Ad vectors. Ad vectors are used primarily to treat benign disorders such as cystic fibrosis [90] but their application has been widened to include cancer treatment [91]. The protocol for Ad vectors generation is found in reference [92].

2.1.1.2 Retrovirus (Rv) vectors [43, 45, 93, 94]

Rv is a class of viruses that can create double-stranded DNA copies of their RNA genomes [95] (Figure 2.2). An example of Rv is human immunodeficiency virus (HIV). Transfection using Rv vectors is via integration of the gene into the chromosome of the target cell. The level of gene expression will be reduced but the gene expression will be longer. A major disadvantage of Rv vectors is that they only transfect proliferating cells since the virus enters the nucleus only at mitosis. The insert-size limit of Rv vectors is 8 kb. Rv is much less immunogenic than Ad as it does not express residual viral genes. The transfection efficiency for most cell types *in vivo* is low because the human complement system can inactivate the virus. There are serious safety concerns about Rv vectors because of the possibilities of integrative inactivation of tumour suppressor genes and RCV generation through recombination with Rvs that are resident in the human genome. Due to these drawbacks, Rv vectors are used in *ex vivo* applications where efficient transfection of cultured cells can be achieved and where sustained gene expression is needed. Another disadvantage of Rv vectors is the difficulty in large-scale production due to RCV. There are also difficulties in achieving high titres and retaining infectivity. In spite of these disadvantages, Rv has been used in more clinical trials than any other vector because of its ability for longer expression. The protocol for Rv vectors generation is found in reference [96].

Figure 2.2. Schematic of retrovirus [97].

2.1.1.3 Adeno-associated virus (AAV) vectors [43, 45, 98, 99]

AAV is a class of small, linear single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19. AAV can efficiently transfect both dividing and non-dividing cells [100]. It is able to give prolonged gene expression *in vivo* compared with other viral vectors; expression of genes has been detected two years after administration in mice [101] and several months after injection in dogs [102], primates [103] and man [104]. This ability to sustain gene expression is a major advantage in treating chronic diseases. The immune response to AAV-transfected cells tends to be weak or undetectable because the vectors contain only short viral sequences. A key drawback of AAV vectors is the low insert-size limit of 4.5 kb. Another major concern is the low titre, contamination and expensive purification procedures during the manufacturing process. Due to these manufacturing challenges, there are only a few clinical protocols involving AAV. The protocol for AAV vectors generation is found in reference [105].

2.1.2 Non-viral gene delivery techniques

Although viral vectors are the most efficient, there is always the fear that the viral vector, once inside the patient, may recover its ability to cause disease. Therefore, there have been major efforts in trying to improve the relatively low transfection efficiency of non-viral vectors.

There are basically two categories of non-viral gene delivery systems, i.e., chemical and physical methods [106]. Cationic lipids and cationic polymers fall under chemical methods, physical methods consist of high pressure delivery methods, electroporation, laser beam gene transduction, ultrasound and photochemical internalization.

2.1.2.1 Chemical methods

The use of lipid-based vectors was first reported in the late 1980s and since then they have been considered to be one of the most promising methods for non-viral gene delivery [55, 106-111]. The cationic head groups make strong electrostatic associations with the nucleic acid, eventually leading to the collapse of the anionic DNA. The length and degree of saturation of the lipid chain is significant in determining the stability and toxicity of the liposome. To form a stable complex, the cationic lipid is often combined with a neutral lipid and/or a helper lipid to form a liposome stable under physiological conditions. Plasmid DNA containing the gene of interest is incorporated into the liposome to form DNA/liposome complexes, or lipoplexes. *Lipid-based vectors, such as Lipofectamine [52, 112], are widely used for in vitro transfection of mammalian cells in research [113]. Although they typically exhibit high in vitro transfection efficiency, there are some drawbacks to the use of Lipofectamine. Major limitations of lipid-based vectors are their need for specific*

ligands to be attached in order to target specific cell types, their inability to transfect non-permissive cells, their longer incubation time and their toxicity in in vivo applications [59, 106, 113].

Cationic polymers condense DNA into compact structures by neutralizing the anionic charge on the DNA [106, 114-121]. The resulting cationic DNA/polymer complexes, or polyplexes, encapsulate the DNA into small particles for gene delivery. Common polycations include polylysine, polyamines such as polyethylenimine, polyarginine-containing proteins, and cationic dendrimers.

2.1.2.2 Physical methods

Physical delivery of naked DNA to cells elicits minimal immune response as compared to DNA encapsulated in lipids or cationic polymers. The lack of immunogenicity of naked DNA makes it a good prospect for gene therapy [43]. The limitations with this approach arise because naked DNA is unprotected against nuclease degradation and DNA does not have target specificity. Thus, the actual physical delivery of naked DNA must be directed towards the tissues of interest since no target ligands are attached to the DNA.

Several methods for the delivery of naked DNA involve the use of high pressure methods to force the DNA into the desired tissues. Particle bombardment methodology (i.e., gene gun) provides a promising mechanism for gene delivery due to the small quantities of DNA required and minimal toxicity [106, 122-124]. To deliver DNA by gene gun, the DNA must first be coated on gold or tungsten microparticles

and then delivered to the cytoplasm of target cells by a pressurized blast from the gene gun (Figure 2.3).

Figure 2.3. Schematic of a gene gun [125].

Another high-pressure method is hydroporation, it delivers DNA to the target by injecting a large volume of solution with significant force (i.e., a burst of DNA-containing solution). Studies have shown that by hydrodynamic injection, delivery vectors were able to reach the target tissue (liver, kidney and muscle) [126-129]. Although increased gene delivery and expression have been observed by this delivery method, it remains a very invasive technique which has been found to increase blood pressure and decrease heart rate due to the volume expansion in the body [106].

Jet injection (Figure 2.4), like hydroporation, delivers a DNA-containing solution to target tissue via a high-pressure mechanism [106, 130]. The development of a novel

low volume 'high speed jet injector' allows for the efficient delivery of small volumes of DNA and is not affected by the length of DNA used. The modified jet injector can penetrate tissues up to 10 mm deep and has displayed transfection efficiencies similar to those observed with particle bombardment techniques. Gene delivery by jet injection is an appealing mechanism because the DNA remains intact throughout the delivery process (i.e., it is not sheared by the pressure) with no tissue damage or immune response observed.

Figure 2.4. Schematic of a jet injector [131].

Electroporation employs the use of an electric field to increase cell permeability and thereby increase the efficiency of DNA uptake into target cells (Figure 2.5). Naked DNA is delivered by needle into the target tissue, which is then stimulated by the application of an electric field. Gene expression by electroporation has been found to be several orders of magnitude higher than injection of naked DNA alone [132]. This

method of gene delivery has been used to target a wide variety of tissues but is particularly useful for delivery to superficial tissues such as skin and muscle. Electroporation of internal tissues such as kidney and liver has shown gene expression, but major limitations exist due to the invasive nature of the delivery (i.e., surgery to apply electric field to the target tissue). Serious tissue damage has been associated with electroporation techniques. Modifications to electroporation mechanisms have been designed to optimize gene uptake while reducing tissue damage introduced by exposure to high electric currents [106, 133-136]. In the case of *in vitro* gene delivery, electroporation has also been known to cause considerable cell death.

Figure 2.5. How electroporation delivers DNA [137].

Laser beam gene transduction (LBGT) may provide a less damaging and less invasive alternative to gene delivery by electroporation [106, 138]. In this method, the injection of naked DNA into target tissue is followed by the direct application of a pulsed femtosecond laser beam for short intervals. The resulting gene expression was comparable to electroporation methods performed under identical conditions. Histological studies revealed that damage was minimal in tissues that received the laser treatment whereas those treated by electroporation showed significant damage.

Thus, this study shows that LBGT may provide a suitable alternative to electroporation for gene delivery to skin tissue.

Ultrasonic gene delivery (Figure 2.6) employs clinical ultrasound techniques to increase the permeability of target tissues and increase uptake of vectors [106, 132, 139, 140]. Both high and low intensity ultrasonic velocities have been found to improve gene expression in a variety of tissues, including muscle, carotid artery, heart and tumors. The use of microparticles for encapsulation of naked DNA is a novel innovation in ultrasonic techniques. DNA is incorporated into gas-filled particles that are generally coated with lipids, polymers or molecules such as albumin. These particles may be targeted to specific tissues by the incorporation of peptides or other target molecules to the surface of the microparticle. This technology is attractive because DNA is protected from degradation and is released from the particles due to ultrasonic energy.

Figure 2.6. Schematic diagram of ultrasonic gene delivery [140].

Photochemical internalization (PCI) relies on light-sensitive release of DNA, which is contained within endosomal vesicles for more efficient gene delivery (Figure 2.7) [106, 141-143]. The cells are incubated with photochemical reagents which associate with endosomes and lysosomes. Exposure to light at the appropriate wavelength activates the reagents and leads to lysis of these intracellular compartments, thereby releasing DNA into the cells. *In vitro* studies involving the delivery of PEI-encapsulated p53 genes to cells resulted in a two to three-fold increase in the expression of the p53 gene [141].

Figure 2.7. Schematic diagram of PCI-mediated gene delivery [143].

2.2 Barriers to gene delivery using non-viral methods

To appreciate the challenges researchers face in improving the low transfection efficiency of non-viral vectors, we should understand what it takes for nucleic acid to be successfully delivered to its target site.

2.2.1 Extracellular barriers [121, 144-146]

For an *in vivo* vector, its mission is to reach the cell surface without being cleared from the body and to protect the nucleic acid from degradation by nucleases. The nucleic acid/vector complex must remain stable in the extracellular fluid, where a high concentration of polyelectrolytes might disrupt this interaction. Polymers with strong cationicity are required because they can bind tightly to the nucleic acid. This is to ensure the nucleic acid/vector complex remains stable in the extracellular fluid where a high concentration of polyelectrolytes exists which might disrupt this interaction. Strong cationicity of the polymer can also produce a compact structure that impedes enzymatic degradation of the nucleic acid. However, too much net positive charge on the surface will cause aggregation and coating by negatively charged proteins such as albumin [114].

2.2.2 Intracellular barriers [121, 144-147]

Once the vector reaches the target cells, they bind with the negatively charged cell membrane and are subsequently internalized. The process of cell surface binding and internalization of the delivery system is known as endocytosis (Figure 2.8).

Figure 2.8. Process of endocytosis [44] (a) Binding of nanoparticles to cell-surface glycoproteins. (b) Binding triggers part of the cellular membrane to envelop the nanoparticle and bring it into the cytoplasm with a membrane-bound vesicle (endosome). (c) Endosomes fuse with other cellular endosomes and mature into degradative lysosomes. (d) Nanoparticles must escape these vesicles to prevent degradation of their therapeutic payload. (e) The material must then release the nucleic acid to perform its therapeutic function in either the cytoplasm, or (f) the genetic material must traverse the nuclear membrane for gene regulation.

After endocytosis, most of the vectors are sequestered in the acidic vesicles within the endosomal compartments, which fuse with the lysosomes. Potent enzymes and low pH in these compartments cause degradation or swelling of the vector, releasing and also breaking down the nucleic acid. A significant step in the gene transfer process is the escape of the vectors or the nucleic acid into the cytoplasm. To enhance gene transfer efficiency, destabilization of the endosomes/lysosomes by endosomolytic reagents such as chloroquine, lipids, and peptides are used [146]. Polymers that have this endosomal disruptive property have figured prominently in design considerations. They have lipophilic side chains or peptide attachment to the side chains to interact specifically with the vesicle membrane. Alternatively, polymers with excessive amino

groups have been hypothesized to reduce acidification of the endosome, eventually causing endosomal swelling and release of the complexes. This “proton sponge” effect was first postulated to explain the effectiveness of polyethylenimine (PEI) in gene delivery [39]. According to this hypothesis, PEI and nucleic acid/PEI complexes in the endosome absorb the protons that are pumped into this organelle. Protonation of the amine groups on PEI leads to swelling of the polymer due to a simultaneous influx of chloride ions to neutralize the build up of a charge gradient. Eventually, an increase in osmotic pressure destabilizes and ruptures the endosome, leading to the release of the complexes into the cytoplasm.

Vectors that escape from the vesicles into the cytoplasm intact can travel to the nuclear membrane via diffusion. There is evidence that vector-containing vesicles can be transported to the nuclear membrane via molecular motors, it is not known what could be used to accelerate this active and passive diffusion process. The vector can also be dissociated to release nucleic acid, which is degraded in both the endosome and the cytoplasm. Nucleic acid directly injected into the cytoplasm is rapidly degraded with an apparent half-life of 50-90 min [148]. The translocation of free nucleic acid from the cytoplasm to the nucleus is likely through diffusion which is a relatively slow process compared with cytoplasmic degradation, this leads to inefficient nuclear transport. Therefore, it is deduced that the nucleic acid would be more stable in the cytoplasm if it exists as a complex with the vector. However, only certain polymers, such as PEI, protect the nucleic acid in the cytoplasm and promote nuclear transport.

Inefficient transfer of nucleic acid from the cytoplasm to the nucleus has been identified as a major factor restricting transgene expression with only 0.1 % of naked

DNA or 1 % of vector-DNA complex reaching the nucleus following microinjection [149]. This process is particularly inefficient in post-mitotic and quiescent cells, the majority of cells *in vivo*, since there is no breakdown of the nuclear envelope in the absence of mitosis. Cellular and viral proteins enter the nucleus efficiently by means of nuclear localization sequences (NLSs), which are stretches of amino acids that bind to intracellular transport receptors such as importins and facilitate transfer through the nuclear pore. The exact conditions to successfully utilize the properties of NLS to enhance non-viral gene transfer, however, require further investigation. It is not clear, e.g., what type, length or number of NLS peptides is best suited for this application. It is also unclear which delivery vectors should be used with NLS peptide/DNA conjugates to exploit the NLS-transport pathways. Previous studies have observed enhanced gene expression with NLS peptide/DNA conjugates using cationic lipids and branched PEI [150]. However, intracellular trafficking and location of DNA release from these types of vectors are thought to differ greatly, with cationic lipids releasing DNA into the cytoplasm and branched PEI/DNA complexes remaining largely intact. Branched PEI might even be expected to hinder NLS activity by masking the peptide and preventing interaction with transport receptors.

2.3 Magnetic transfection

Magnetic transfection is a recently developed non-viral gene delivery technique which uses physical forces to enhance gene transfection. It was first reported by Mah *et al* in 2000 [151]. Essentially, this enhancement comes from overcoming the limitation in current delivery systems of insufficient contact of the vector with target cells [53].

2.3.1 Benefits of magnetic transfection

Mah and co-workers used magnetic microspheres conjugated to recombinant adeno-associated virus 2 (rAAV) in their *in vitro* and *in vivo* studies [152]. From their *in vitro* study using C12S cells, they found that transduction was greater with vectors bound to magnetic microspheres (76 %) compared to those bound to polystyrene microspheres (59 %). They also reported that just 1 % of vector conjugated to magnetic microspheres could achieve the same level of transduction as those achieved with the free vector. *In vivo* studies on adult 129/Svj mice showed that transduction efficiency was also higher with magnetic microsphere assisted delivery compared to using only free vectors. They hypothesized that the higher transduction efficiency was due to a higher concentration of the vector and longer period of exposure of the vector to the target cells. The ability to target specific regions for infection was also demonstrated in their work.

Plank's group followed up with a more detailed study, they are the first to term magnetic particle assisted gene delivery as magnetofection (short for magnetic transfection) [17]. For their *in vitro* and *in vivo* studies, they associated gene vectors, e.g., commercial formulations such as Lipofectamine and GenePorter, recombinant adenovirus and retrovirus with superparamagnetic iron oxide nanoparticles (SPION) coated with PEI (800 kDa). These vector-magnetic particle suspensions have particle sizes in the range of 400 to 1000 nm. Enhanced transfection of up to three orders of magnitude compared to vectors without magnetic particles was reported in their *in vitro* study using NIH3T3 and CHO cells. Gene delivery was confined to a region defined by the dimension of the external magnet. Peak transfection levels were achieved within 10 min of incubation time compared to the 2 to 4 h for standard

transfection without magnetic particles. Lower vector dose was needed with magnetic particles to achieve similar level of gene expression. Successful gene delivery to nonpermissive cells such as NIH3T3, K562 and primary human peripheral blood lymphocytes, PBL, were also demonstrated. Efficient gene delivery was also observed in the gastrointestinal tract and in blood vessels of Wistar rats and B16/Cico mice.

Krotz *et al* investigated magnetofection in human umbilical vein endothelial cells (HUVEC), which is virtually resistant to efficient transfection [59], commercial magnetic beads (CombiMAG) were used. Transfection efficiency was increased up to 360-fold for magnetofection compared to Lipofectamine, a commercial cationic lipid transfection reagent. There was only a 1.6-fold increase in toxicity caused by magnetofection.

Gersting *et al* examined the ability of magnetofection in an *ex vivo* airway epithelium organ model [58]. In topical gene delivery application in airways, a major barrier is the short contact time of complexes with the cell surface due to mucociliary clearance. Commercial superparamagnetic nanoparticles coated with PEI (transMAG-PEI) were used. It was found that transfection efficiency was significantly enhanced, gene expression via magnetofection was 400-fold higher compared to PEI-mediated transfection and 2500-fold higher compared to Lipofectamine-mediated transfection. Magnetofection resulted in efficient gene delivery after 5 min of incubation time, a high level of expression was detected after 15 min of incubation time compared to 4 h for PEI-mediated transfection. This is useful for *in vivo* applications where long incubation time at the target tissue is not practical. The impact of magnetic field was

also studied, application of magnetic field resulted in enhanced transfection efficiency by up to two orders of magnitude.

Bhattarai *et al* examined the use of magnetic particles for viral gene delivery in K562 cells (human leukemia cells), a suspension cell line [153]. Cationic chitosan-coated iron oxide nanoparticles (50 - 70 nm in diameter and zeta potential of +20 mV) were associated with adenoviral vectors for magnetofection. The viral gene expression was up to 100 times larger with magnetic particles as compared to only the viral vector. Commercial magnetic particles (50 - 150 nm and zeta potential of -22 mV) were also used as a comparison and they did not show enhanced gene expression. It was suggested that enhanced gene expression by cationic magnetic particles was due to their ability to interact with the negative charge of negatively charged proteins on viruses.

Lee *et al* reported the first use of magnetofection in embryonic stem (ES) cells [154]. Commercial magnetic particles (PolyMAG) and a conventional lipid-based transfection reagent (FuGENE 6) were used. Magnetofection resulted in higher transfection efficiency (45 %) compared to the FuGENE 6 method (15 %).

Mair *et al* prepared 200 nm size iron nanoparticles by a physical vapour deposition technique [155]. This technique allows various alloy compositions and concentrations to be made, enabling the properties of the particles to be tuned, it is also possible to create particles with complex shapes and dimensions as small as 40 nm. *In vitro* magnetofection in a modified HeLa cell line showed enhanced gene expression of 76 % compared to Lipofectamine and 139 % enhancement compared to magnetic particles delivered without an external magnetic field.

Shi *et al* used magnetic gene vectors with hyperbranched PEI (HPEI) of 110 kDa and 160 kDa in their *in vitro* study on COS-7 cells [156]. These particles were about 3 nm in diameter with a saturation magnetization of 46 emu/g for HPEI160k coated particles and 40 emu/g for HPEI110k coated particles. Transfection efficiency for HPEI110k-magnetic gene vector was four times higher than standard HPEI110k transfection. Transfection efficiency for HPEI160k-magnetic gene vector was 1.5 times higher than standard HPEI160k transfection. The higher transfection efficiency of HPEI110k-magnetic gene vector was attributed to its larger complex size, which caused more complexes to be concentrated on the target cells. The effect of additional free HPEI was also studied, with additional HPEI (N/P = 10), gene expression for HPEI110k-magnetic gene vector was 13 times higher than standard HPEI110k transfection; for the HPEI160k-magnetic gene vector, the enhancement was seven times that of standard HPEI160k transfection. The HPEI110k-magnetic gene vector has lower cytotoxicity than the HPEI160k-magnetic gene vector.

Namgung *et al* used SPION coated with polyethylene glycol and branched PEI (1.8 kDa) in their work [157]. The diameters of the uncoated SPION and coated SPION were 5 nm and 10 nm respectively. The saturation magnetization of the coated SPION decreased significantly to 10 emu/g from 40 emu/g after coating. The size of the complex, determined from TEM analysis, was 50 nm. However, the hydrodynamic diameter was not determined. Thus, it is unclear if the complex size was 50 nm during magnetofection. The zeta potential of the coated SPION was +10 mV. The cell viability study showed no significant cell toxicity for these particles (N/P = 10) in NIH3T3 cells, however, the viability was 80 % in PC-3 cells. The transfection

efficiency of BPEI(1.8 k) coated SPION was 50 % higher than that of BPEI (25 kDa) in PC-3 cells, gene expression was 15 times higher for samples under a magnetic field (15 min incubation time) compared to samples not subjected to a magnetic field. Additional incubation time of 3 h, following removal of the suspended complexes after the initial 15 min incubation time, did not enhance transfection. It was hypothesized that magnetofection resulted in rapid sedimentation of the magnetic complexes onto the cell surface. The higher concentration of complexes on the surface is expected to result in more complexes inside the cell and hence inside the nucleus.

2.3.2 Factors that affect magnetic transfection

2.3.2.1 Incubation time

Plank *et al* examined the transfection kinetics of magnetofection in NIH3T3 cells using lipofection reagents Lipofectamine and GenePorter associated with PEI-coated magnetic nanoparticles [69]. Incubation times of 5, 10, 20, 40 and 240 min, in the presence of a magnetic field, were used. They found a logarithmic relationship between transfection efficiency and incubation time for GenePorter. For Lipofectamine, the transfection efficiency decreases with increasing incubation time. For standard transfection using only lipofection reagents, the relationship is linear. Magnetofection with PEI and DOTAP-Cholesterol also yielded a logarithmic profile. Maximum transfection efficiency could be achieved within 5 to 20 min of incubation time.

Li *et al* suggested that a maximum of 30 min of incubation time under magnetic field was sufficient to achieve maximum transfection efficiency [158]. In their work, NIH3T3, HEK293 and COS-7 cells and incubation times of 7, 15, 30 and 60 min were

studied. Branched PEI (25 kDa)-coated commercial magnetic nanobeads (MNB) of average size of 200 nm were used.

These studies showed that transfection efficiency has a logarithmic relationship with incubation time under magnetic field. Maximum transfection efficiency could be achieved within 5 to 30 min of incubation time.

2.3.2.2 Hydrodynamic diameter and N/P ratio

Chorny *et al* studied the effect of magnetic particle size on transfection efficiency. They used polylactide MNP coated with PEI on rat aortic smooth muscle cells (A10) and bovine aortic endothelial cells (BAEC) [31]. MNP with 375 nm hydrodynamic diameter resulted in transfection efficiency 1.8 to 2.7 times higher than MNP of smaller sizes (185 nm and 240 nm) in A10 and BAEC respectively. The higher transfection efficiency of larger sized MNP was attributed to small colocalization in the lysosomes.

Wang and co-workers also examined the effect of aggregate size on transfection efficiency in NIH 3T3 cells [75]. The aggregate size of the PEI-coated MNP was controlled by varying the ratio of PEI to MNP. For the same PEI to MNP ratio, the aggregate size could be further reduced by acidification and subsequent neutralization. For PEI to MNP ratios of 1.2, 1.6 and 2, the hydrodynamic diameters of PEI-MNP were 380, 230 and 200 nm. After complexing with DNA, the sizes were 5000, 1000 and 700 nm. The transfection efficiency of 200 nm PEI-MNP was ~ seven times higher than that of 380 nm PEI-MNP. The increased transfection efficiency could also be due to the higher amount of PEI instead of solely due to the smaller aggregate size.

For a PEI to MNP ratio of 2, after acidification and subsequent neutralization, the aggregate size reduced from 200 nm to 75 nm. This resulted in an increase of 20% in transfection efficiency although interestingly, there was no statistically significant difference in the size of the complexes.

The findings regarding the effect of aggregate size on transfection efficiency from both studies were conflicting. Chorny *et al* reported larger aggregate size MNP resulted in higher transfection efficiency whereas Wang *et al* reported otherwise. In the latter study, the higher transfection efficiency could be attributed to higher N/P ratio as there was more PEI on the smaller size aggregate. Higher N/P ratio in turn affects the amount of complexed DNA, its compaction and release.

2.3.2.3 Magnetic particle size and magnetic field strength

Furlani and Ng modeled *in vitro* magnetofection using the drift-diffusion equation and used it to predict the rate of particle accumulation as a function of particle radius and magnetic field gradient [159]. Such a model provides useful insight to optimize the performance of magnetofection. They found that the magnetic particles should be in the size range of 100 to 1000 nm in diameter for effective magnetofection. Also, the smaller the separation between the magnet and the chamber, the faster will be the rate of particle accumulation.

This modeling study showed that larger individual particle size (hence higher saturation magnetization and susceptibility) and higher magnetic field strength result in more complexes being accumulated on the cells, which will in turn result in higher transfection efficiency.

2.3.2.4 Pulsed magnetic field

To further enhance the efficiency of magnetofection, Kamau *et al* applied a pulsed (oscillating) magnetic field generated by an electromagnet after the static magnetic field has sedimented the MNP onto the cells [30]. They synthesized 50 nm PEI coated iron oxide MNP and also utilized commercial polyMAG (200 nm) in their work, transfection efficiency in HeLa, 293T, COS-7 cells and synoviocytes were studied. In their experiments, a permanent magnet was placed below the culture plate for 20 min followed by 5 min application of a pulsed magnetic field. The permanent magnet generated a magnetic flux density of 250 mT in the area of the cells with a magnetic field gradient of 10 T/m. The pulsed magnetic field which has a maximum amplitude of 27 mT and a field gradient of 10 T/m, had a frequency of 50 Hz and 0.75 Hz perpendicular and parallel to the surface of the well, respectively. The transfection efficiency doubled with the application of pulsed magnetic field compared to cells exposed only to a static magnetic field. This increase was attributed to the oscillating movements of the magnetic particles on the cell surface which may help in the crossing of the cell membrane. In another study which was performed on primary cell lines which are known to be more difficult to transfect, significant enhancement due to the pulsed magnetic field was observed in chondrocytes, osteoblasts, melanocytes and lung fibroblasts [160].

McBain and co-workers also used oscillating magnetic fields in their work [77]. The major difference between their work and that of Kamau [30] is that the stronger oscillating magnetic field of ~100 mT at the cell surface was generated by an array of permanent magnets moving at frequencies of 1, 2 and 4 Hz. Commercial MNP,

PolyMAG, and human lung epithelial (NCI-H292) cells were used in this work. A frequency of 2 Hz with an amplitude of 200 μ m produced the best results, transfection efficiency was ~ 100 % better than that of the static magnetic field. It was postulated that the horizontal movement of magnetic complexes on the cell surface stimulated the cell membrane, which promotes more efficient endocytosis.

Chen *et al* used a strong pulsed magnetic field of 0.6 T to effect fast transfection of adherent (HeLa) and suspended (HeLa and CHO) cells [161]. Iron oxide nanoparticles of 10 nm in diameter coated with PEI (750 kDa) were used. During transfection, impulsed magnetic field lasting 3 ms were applied several times with a 6 s break between each pulse. The transfection efficiency was doubled compared to cells not exposed to a magnetic field. Transfection efficiency was higher (60.7 %) compared to standard magnetofection (52.4 %) employing a static magnetic field of 250 mT. Cell viability was also double that of standard magnetofection. The mechanism was suggested to be magnetic “bombardment” as well as cell endocytosis since the magnetic complexes were found to be internalized in the cytosol of cells after exposure to the impulsed magnetic field.

These studies demonstrated that magnetic transfection efficiency could be enhanced by the use of pulsed magnetic field. The increase in transfection efficiency was postulated to be due to the movement of the complexes along/across the cell surface.

2.3.3 Mechanism of magnetic transfection

In their transfection experiments, Huth *et al* investigated the mechanism of magnetofection using electron microscopy and endocytosis inhibitors [72]. The

objective is to determine whether the magnetic field caused a direct uptake of DNA/MNP complexes into the cell. TransMAG, a commercial PEI-coated MNP of average size of 200 nm was used. Transfection experiments were done on HeLa, BEAS-2B and HEP-G2 cells. A cellular trafficking study using electron microscopy showed that there is no difference between the transMAG particles and transMAG-DNA complexes. A transfection study using endocytosis inhibitors in three different cell lines revealed that endocytosis inhibition was similar for PEI-mediated transfection and magnetofection in each cell line. A study of the effect of magnetic field on endocytosis showed that application of magnetic field till the point of assay did not result in significant difference in transfection efficiency. In short, this study on the mechanism of magnetofection showed that magnetic force did not cause circumvention of the endosomal pathway, instead, it resulted in rapid sedimentation of complexes on the cell surface.

Sauer *et al* studied the mechanism of magnetofection by tracking the complex internalization and intracellular movement in real time via a highly sensitive custom-built fluorescence microscope [162]. A commercial cationic lipid transfection reagent Metafectene Pro was used together with Fluomag-C, a commercial polymer coated iron oxide magnetic particles, to deliver DNA to HuH7 cells. The complexes were 388 nm in diameter and have a zeta potential of 41.3 mV. They found that the mechanism for magnetic complexes and non-magnetic complexes are very similar. Both consist of three stages; stage I where complexes attached to cell surface and showed slow transport behaviour, stage II where complexes exhibit confined diffusion and stage III where active transport along microtubules occurs. The study found that internalization took about 1 h by endocytosis and DNA was not separated from the

magnetic particles after 24 h (time of assay). It was also observed that non-magnetic complexes detached after reaching the cell surface, whereas such detachment was not observed for magnetic complexes. Thus, it was concluded that enhanced transfection efficiency in magnetofection was due to higher amount of complexes on the cell surface.

Smith and coworkers used caveolin and clathrin immunofluorescence to study the type of endocytosis that occurs during MNP uptake [163]. Gold coated iron oxide nanoparticles of 6 nm and 500 nm in diameter, functionalized with HIV1-tat peptide were used to study cellular uptake in Infinity Telomerase Immortalised primary human fibroblasts (hTERT-BJ1) cells. It was found that smaller 6 nm MNP uptake was via caveolin mediated endocytosis and clathrin endocytosis was involved in 500 nm MNP uptake.

Ang *et al* synthesized 8, 16 and 30 nm iron oxide MNP coated with PEI for their study on the mechanism of magnetofection in COS-7 cells [164]. It was hypothesized that the plasma membrane of the cell would be disrupted if MNP are pulled directly into the cell during magnetofection. Cell wounding assays suggest that the mechanism of magnetofection is not cell wounding.

Stride *et al* combined ultrasonic mediated transfection with magnetofection [165]. Phospholipid coated bubbles mixed with magnetic micelles of mean diameter 2 μ m were added to Chinese hamster ovary (CHO) cells in a medium containing plasmid DNA. Exposure to ultrasound and magnetic field resulted in a three-fold increase in transfection efficiency compared to magnetofection alone. It was proposed that the

cells could be sensitised by the presence of the micelles, which made them easier to be transfected during their exposure to ultrasound.

Song *et al* incorporated cell-penetrating Tat peptide to the standard magnetofection technique to further enhance transfection efficiency [166]. Tat is a highly cationic peptide which has the ability to transport particles across plasma and nuclear membranes. Ternary complexes comprises polyMAG, DNA and Tat peptide were used for *in vitro* transfection of the human astrocytoma cell line U251 and neural cell lines including human glioblastoma U87 cells, mouse C17.2 neural stem cells and human NT2 neural stem cells and *in vivo* transfection in rat spinal cord. These ternary complexes have an average hydrodynamic diameter of 120 nm and zeta potential of +50 mV. Transfection efficiency improved by up to four times in U251 cells compared to binary complexes (complexes without Tat peptide). There was also significantly higher level of gene expression in the other cell lines. Addition of Tat peptide to the complex did not significantly affect the cell viability. The *in vivo* transfection study showed that transfection efficiency using ternary complexes was double that of binary complexes.

2.4 Magnetic vectors for magnetic transfection

The magnetic vectors used in magnetofection consist of a magnetic core coated with a cationic polymer. The reason for coating with a cationic polymer is to functionalize the particles so that they are able to electrostatically bind to the anionic DNA molecules. The rationale for using SPION is because they are approved for use in humans [167]. Clinically, SPION has been used for hyperthermia, radio therapy, magnetic resonance imaging (MRI) and gene delivery. Dextran, anhydroglucose or

carbonate coated SPION of size 100-1000 nm exhibit excellent biocompatibility in clinical tests [168].

2.4.1 Magnetic behaviour of superparamagnetic nanoparticles

Nanoparticles that are made of ferro- or ferrimagnetic material, and below a certain size (generally 10-20nm), can exhibit a unique form of magnetization called superparamagnetism. Superparamagnetic nanoparticles have weak magnetostatic interaction because their individual moments fluctuate strongly. When the external magnetic field is removed, due to thermal energy there is no net magnetization. This property is useful for *in vivo* biomedical applications since aggregation of magnetic particles could be harmful. These particles can possess significant saturation magnetization [169]. A typical magnetization curve for superparamagnetic nanoparticles is shown in Figure 2.9 [27].

Figure 2.9. M-H curve: Magnetization vs applied magnetic field [27].

The Neel relaxation time τ of the net magnetization of the superparamagnetic particle is given by [15]:

$$\tau = \tau_0 \exp\left(\frac{KV_M}{k_B T}\right) \quad (\text{Eq. 2.1})$$

where τ_0 is the attempt time, K is the anisotropy energy density, V_M is the particle volume, k_B is the Boltzmann constant, and T is the absolute temperature. For non-interacting particles, τ_0 is of the order of 10^{-10} to 10^{-12} s.

Superparamagnetism does not just depend on the temperature, but also on the measurement time τ_m of the experimental technique. If $\tau \ll \tau_m$, the reversal of moment is fast relative to the measurement time, the particle will appear to be paramagnetic. If $\tau \gg \tau_m$, the reversal is slow and quasi-static properties will be observed. In typical experiments, τ_m can range from 10^2 s for DC magnetization and 10^{-1} - 10^{-5} s for AC magnetization.

2.4.2 Synthesis of iron oxide MNP

There are various methods of synthesising iron oxide nanoparticles [170]. Commonly used techniques include coprecipitation, reactions in constrained environments (such as reverse micelle), hydrothermal reactions, sol-gel reactions and polyol methods.

The coprecipitation method is probably the easiest way to synthesize reasonably homogeneous iron oxide nanoparticles [171-173]. Magnetite is obtained by aging stoichiometric mixture of ferrous and ferric salts in aqueous medium. The chemical reaction is as shown in equation 1.1.

Thermodynamics dictates that magnetite would be obtained in a non-oxidizing environment at a stoichiometric ratio of $\text{Fe}^{2+}/\text{Fe}^{3+}$ equal to 1:2 in a pH range of 8 and 14 [174]. The magnetic properties of the particle are strongly dependent on its size, studies have shown that the particle size can be controlled by varying pH, ionic strength and synthesis temperature [172, 175, 176].

Synthesis of iron oxide via reverse micelle method involves aqueous iron salt solutions being encapsulated by surfactant molecules which separate them from a surrounding organic solution [177-179]. This constraint environment limits particle nucleation and growth. The size of the particle can be controlled by the temperature and surfactant concentration [180]. Inexpensive large-scale synthesis of uniform and crystalline magnetite nanoparticles could be obtained using this technology [181].

The hydrothermal reaction method involves thermal decomposition of ferrous salts with organic solvents performed in autoclaves where pressure can go above 2000 psi and temperature above 200 °C [182, 183]. The particle size depends on the water content, reaction time and temperature [184]. A high level of monodispersity and size control can be achieved by this method.

The sol-gel synthesis method is based on the hydroxylation and condensation of molecular precursors in solution at room temperature which will result in a three-dimensional metal oxide network denominated gel, the gel is subsequently heated to obtain the iron oxide nanoparticles [185, 186]. The parameters that affect the structure

and properties are solvent, temperature, nature and concentration of the salt precursors, pH and agitation [187, 188].

The polyol method is similar to sol-gel except that the solvents used are polyols [189, 190]. Polyols have high boiling points, thus offering a wider operating temperature range. Polyols also function as stabilizers to control particle size and prevent particle aggregation [191].

2.4.3 PEI coating

PEI belongs to a promising class of non-viral gene vector known as cationic polymer. PEI is one of the most efficient non-viral vectors for *in vitro* and *in vivo* gene delivery [39, 192, 193]. PEI is highly water-soluble and consists of repeated units of nitrogen atom and amino groups that allows protonation to occur (Figure 2.10).

Figure 2.10. Structure of branched PEI [192].

The pKa of the primary amines is around 5.5 [115]. In physiological conditions, about 20% of the nitrogens are protonated [194]. The positive charges on PEI give it the ability to compact the relatively large negatively charged DNA with hydrodynamic diameter of hundred of nanometers to only tens of nanometers, thus facilitating its uptake into cells [195, 196]. Figure 2.11 shows the electrostatic interaction between the amine groups of PEI and phosphate backbone of DNA [34]. This compaction process also protects the DNA from degradation by serum nucleases under *in vivo* conditions and from enzymes inside the cell [195, 197]. The transfection efficiency of PEI is a function of its molecular weight, geometry (linear or branched) and cell type [198-200]. *In vitro* and *in vivo* studies have shown that 25 kDa PEI transfects better than PEI of other molecular weights [39, 199, 201]. It was reported that 25 kDa branched PEI showed the highest transfection efficiency in COS-7 cells [199].

Figure 2.11. Electrostatic interaction between positively charged amine groups of PEI and negatively charged phosphate backbone of DNA [34].

The mechanism of PEI mediated transfection is illustrated in Figure 2.12. The PEI/DNA complexes (polyplexes) accumulate on the plasma membrane of the cell. They enter the cell via endocytosis and are trapped within endosomes. It is generally assumed that these endosomes will merge with lysosomes (forming endolysosomes) for degradation. The effectiveness of PEI as a gene vector is thought to be due to its buffering ability within lysosomes. At physiological pH (7.3), nitrogen atoms on PEI are only partially protonated, thus PEI can accept the proton influx into the endolysosomes. This would result in increased endolysosomal pH, which affects protein folding, thus deactivating the degradative enzymes. At the same time, due to the simultaneous influx of chloride anions, osmotic swelling occurs, which eventually bursts the endolysosomes before DNA degradation can take place. This process is known as the “proton sponge” effect [192]. After endolysosomal disruption, the polyplexes enter the nucleus without separation of DNA and PEI [202]. A study by Godbey *et al* found that PEI offers a stable protective environment for DNA and there was also no lysosomal involvement. The polyplexes moved through the cytoplasm to the nucleus without endolysosome formation [203].

Figure 2.12. Schematic representation of PEI mediated DNA uptake by cell [200]. DNA is compacted by PEI into a polyplex. The polyplex is internalized within endosome upon contact with the cell membrane. The polyplex is released via a “proton sponge” effect which causes an osmotic opening of the endosome. The polyplex then proceeds to the nucleus.

2.4.4 Magnetic particle transport

The motion of a spherical magnetic particle in a viscous fluid under the influence of an applied magnetic field is given by [204, 205]:

$$m_p \frac{d\mathbf{v}_p}{dt} = \mathbf{F}_m + \mathbf{F}_f + \mathbf{F}_g \quad (\text{Eq. 2.1})$$

where $\mathbf{v}_p$ is the velocity of the particle, and $\mathbf{F}_m$, $\mathbf{F}_f$, and $\mathbf{F}_g$ are the magnetic, fluidic, and gravitational forces, respectively. Particles are assumed to be in low concentration, hence particle/fluid interactions and interparticle effects are neglected. This equation also does not take into account Brownian motion as Furlani has shown that this effect is only significant for particle less than 10 nm in diameter [159]. The inertial term m_p is ignored for submicron particles as their mass is negligible. Thus, equation 2.1 becomes:

$$(\text{Eq. 2.2})$$

$$\mathbf{F}_m + \mathbf{F}_f + \mathbf{F}_g = 0$$

The force needed to move a spherical particle through a fluid at a certain velocity ($\mathbf{F}_f$) is given by Stokes' law,

$$\mathbf{F}_f = -6\pi\eta R_{p,hyd} (\mathbf{v}_p - \mathbf{v}_f) \quad (\text{Eq. 2.3})$$

where η is the fluid viscosity, $R_{p,hyd}$ is the hydrodynamic radius of the particle, $\mathbf{v}_p$ and $\mathbf{v}_f$ are the particle and fluid velocity, respectively.

The particle experiences a translational magnetic force ($\mathbf{F}_m$) in the presence of a magnetic field gradient given by [15]:

$$\mathbf{F}_m = \frac{V_p(\chi_p - \chi_f)}{\mu_0} (\mathbf{B} \cdot \nabla) \mathbf{B} \quad (\text{Eq. 2.4})$$

where μ_0 is the magnetic permeability of free space ($1.26 \times 10^{-6} \text{ NA}^{-2}$), V_p is the volume of the particle, $\mathbf{B}$ is the magnetic flux density, χ_p and χ_f are the magnetic susceptibilities of the particle and fluid, respectively.

In an alternating magnetic field, the magnetic susceptibility is a function of frequency [206]:

$$\chi = \frac{\chi_0}{1 + i\omega\tau} = \frac{\chi_0}{1 + (\omega\tau)^2} + \frac{\omega\tau\chi_0}{1 + (\omega\tau)^2} \quad (\text{Eq. 2.5})$$

where χ_0 = susceptibility in a static magnetic field;

$\omega = 2\pi f$, f = frequency;

τ = relaxation time.

At high frequency ($\sim 1 \text{ MHz}$), the magnetic susceptibility of a 10 nm particle is about $0.5\chi_0$. Thus, the magnetic force would be halved.

The above equations will be used in our model detailed in Section 3.5.6.4 to estimate the longest time taken for the DNA/PEI-AMNP complexes to settle on the cells and to determine whether the complex penetrates the cell during the 20 min of incubation under magnetic field.

2.4.5 Mechanism of cell wounding

As mentioned in Section 2.3.2.4, pulsed magnetic field has been reported to increase transfection efficiency [30, 160]. The motion of a MNP acted upon by a pulsed magnetic field is analogous to that of a jackhammer. Such a ‘drilling’ effect, especially under a high magnetic field, may cause the MNP to penetrate the plasma membrane and effect faster and higher transfection rate compared to the slower mechanism of endocytosis. DNA degradation would also be reduced since the magnetic complex can bypass the endocytosis process. Penetration of the plasma membrane could also be possible under a sufficiently high static magnetic field. From equation 2.4, the magnetic force is proportional to the magnetic susceptibility and size (since volume = $\frac{4}{3}\pi r^3$). Since the saturation magnetisation is also proportional to the particle size [207], MNP with higher magnetic properties will give rise to a larger magnetic force and higher possibility of penetrating the plasma membrane.

2.5 Summary

Through these background findings, PEI coated iron oxide MNP was selected to be the transfection vector. Experimental studies showed that incubation time, hydrodynamic diameter, N/P ratio, zeta potential and type of magnetic field have significant effect on transfection efficiency. Modeling study revealed the importance of magnetic

properties and magnetic field strength, but these parameters have not been studied empirically. Hence, all these physical parameters were chosen to be studied in this project experimentally. Previous mechanism studies were performed from the perspective of endocytosis. In this project, the approach was to study the mechanism from the perspective of cell wounding.

CHAPTER 3: MATERIALS AND METHODS

3.1 Outline

This chapter details the materials and experimental procedures for the synthesis of polymer-coated iron oxide nanoparticles and characterization techniques used in this study. Iron (II,III) oxide was synthesized using the alkaline co-precipitation technique. It was subsequently coated with polyethylenimine (PEI). The PEI coated iron oxide was then diluted in nano-pure water and used for transfection in COS-7 cells. Culturing, transfections and assays of COS-7 cells were performed in the Malaria laboratory of the School of Biological Sciences. The protocol for plasmid preparation is listed in appendix B. Characterization of the uncoated and coated iron oxide employed transmission electron microscopy (TEM), vibrating sample magnetometry (VSM), X-ray diffractometry (XRD), thermal gravimetric analysis (TGA) and photon correlation spectroscopy methods.

3.2 Materials

pEGFP-N1 (Clontech Laboratories), pMIR-REPORT and COS-7 cells (kindly provided by Profs. M. S. Featherstone and A. Law of the School of Biological Sciences, Nanyang Technological University, Singapore), Roswell Park Memorial Institute 1640 medium (HyClone), fetal bovine serum (HyClone), penicillin/streptomycin (PAA) and L-glutamine (Gibco) were used in the experiments. Crystal violet (Merck), sodium dodecyl sulfate (Hoefer), PEI 25 kDa, branched (Sigma-Aldrich), ZONYL FSA (Sigma-Aldrich), Spectra/Por 6 50 kDa cut-off dialysis

membrane (Spectrum Laboratories), MagnetoFACTOR plate (Chemicell) (arrays of 96 cylindrical magnets, remanence $B_r = 1.1$ T, in the same geometry as a 96-well plate), Lipofectamine 2000 transfection reagent (Invitrogen), trypsin (Gibco) and luciferase assay kit (Promega) were also utilized. PolyMAG, a commonly used commercial magnetic particle formulation, was obtained from Chemicell. Cell-impermeant tracer fluorescein isothiocyanate (FITC)-dextran (10,000 MW) and cytosolic fluorescent marker Green Cell Tracker were purchased from Molecular Probes. Oregon Green 488 carboxylic acid, succinimidyl ester (Molecular Probes) and Label IT Tracker TM-Rhodamine Kit (Mirus) were used to label PEI-coated MNPs and pEGFP-N1 respectively for confocal laser scanning microscopy studies. Nano-pure water (18.2 M Ω /cm) was obtained from Milli-Q Synthesis (Millipore).

3.3 Nomenclature

The nomenclature of the synthesized magnetic nanoparticles used in this work is as follows:

MNPs: magnetic nanoparticles.

AMNPs: aggregate of magnetic nanoparticles. For example, an individual magnetic nanoparticle is 8 nm in diameter, the aggregate of magnetic nanoparticles of 8 nm in diameter (referred to as A8MNP) has a hydrodynamic diameter of 643 nm. Aggregates of magnetic nanoparticles of 16 nm and 30 nm in diameter are referred to as A16MNP and A30MNP, respectively.

PEI-AMNPs: PEI coated aggregated magnetic nanoparticles. For example, PEI coated aggregate of magnetic nanoparticles of 8 nm in diameter (referred to as PEI-A8MNP) has a hydrodynamic diameter of 647 nm. PEI coated aggregate of magnetic

nanoparticles of 16 nm and 30 nm in diameter are referred to as PEI-A16MNP and PEI-A30MNP, respectively.

3.4 Synthesis and characterization of MNPs

Iron oxide nanoparticles were synthesized as described below using adaptations of the alkaline co-precipitation technique [171-173] and subsequently coated with 25 kDa branched PEI. Three methods were used for the synthesis of the MNPs. The PEI-coated AMNPs were then diluted in nano-pure water and used for subsequent transfection.

3.4.1 Preparation of 8 nm iron oxide nanoparticles

8 nm MNPs were synthesized adapting the technique of Kim *et al* [171]. In a typical experiment, an iron source consisting of 1.28 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (4.32 g), 0.64 M $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1.59 g) and 0.4 M HCl (12.5 ml) was prepared by dissolving the chemicals in nano-pure water. The iron source was added to a 1.5 M NaOH solution (7.5 g NaOH in 125 ml nano-pure water) under bubbling N_2 gas and vigorously stirred at room temperature for 30 min. The precipitated iron oxide was isolated by a permanent magnet (1.2 T) and the supernatant was discarded. After washing the precipitate several times, it was dispersed in nano-pure water, the total volume was made up to 80 ml.

3.4.2 Preparation of 16 nm iron oxide nanoparticles

16 nm MNPs were synthesized by the technique of Nishio *et al* [172]. An iron source consisting of 0.1 M $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ was prepared by dissolving 1.29 g of the chemical in

64.8 ml of nano-pure water and added into an alkaline solution made up of 1.232 l of 0.02 M NaOH (1.03 g) and 8.8 mM NaNO₃ (1.95 g), under bubbling N₂ gas and vigorously stirred at 14 °C for 30 min. The resulting suspension was kept at 4 °C for 24 h after which the AMNPs were isolated using the method described in section 3.4.1.

3.4.3 Preparation of 30 nm iron oxide nanoparticles

The synthesis of 30 nm MNPs was adapted from Nedkov *et al* [173]. An iron source consisting of 0.03 M FeCl₂·4H₂O was prepared by dissolving 3.093 g of the chemical in 519 ml of nano-pure water. This iron source was added into 104 ml of 0.3 M NaOH (1.245 g) solution under bubbling N₂ gas and vigorously stirred at room temperature for 30 min. The resulting suspension was kept at 4 °C for 24 h after which the AMNPs were isolated from the suspension as described in section 3.4.1.

Table 3.1 summarizes the synthesis conditions described in sections 3.4.1-3.4.3.

Table 3.1. Synthesis conditions and properties of 8 nm, 16 nm and 30 nm MNPs.

Sample	Volume ratio of NaNO ₃ : FeCl ₂ ·4H ₂ O	Synthesis temperature (°C)	Aging conditions
8 nm MNP	0:1	25	-
16 nm MNP	5:1	14	24 h, 4 °C
30 nm MNP	0:1	25	24 h, 4 °C

3.4.4 Coating of AMNPs with PEI

The coating procedure followed that of Mykhaylyk *et al* [208]. The coating solution, consisting of 1 g PEI and 0.5 ml ZONYL FSA in 18.5 ml nano-pure water, was added to the iron oxide suspension and stirred for 2 h. The product was sonicated for 10 min

(60 s sonication/30 s break interval) to disperse the AMNPs and then dialyzed against nano-pure water over 2 days using a Spectra/Por 6 50 kDa cut-off dialysis membrane to neutralize the suspension and to remove excess unbound coating material. Finally the PEI-coated AMNPs suspension was diluted to 0.05 $\mu\text{g Fe}/\mu\text{l}$ and kept in aliquots at 4 °C until further use.

3.4.5 Characterization of MNPs

3.4.5.1 TEM analysis

A transmission Electron Microscope (JEOL JEM 2010) operated at 200 kV was used to determine the particle morphology, size and distribution.

3.4.5.2 XRD analysis

A X-ray diffractometer (Shimadzu 6000) operated at 50 kV and 50 mA with monochromatic Cu-K α radiation ($\lambda = 0.154$ nm), scanning speed of 2 °/min and scan range from 10 ° to 80 ° was used to determine the phase of iron oxide.

3.4.5.3 Magnetic properties

A vibrating sample magnetometer (Lakeshore 7400) operated at ± 10 kG at room temperature was used to determine magnetic properties such as saturation magnetization (Ms) and magnetic susceptibility (χ).

3.4.5.4 Hydrodynamic diameter and zeta potential

Photon correlation spectroscopy (Malvern Zetasizer Nano ZS) was used to determine the aggregate size and zeta potential of the AMNPs dispersed in phosphate buffered saline (PBS), pH 7.4, at a concentration of 0.01 $\mu\text{g}/\mu\text{l}$. Measurements were performed

at room temperature with ten sub-run measurements per sample and Henry's Function $F(Ka)$ equal to 1.5 (Smoluchowski approximation).

3.4.5.5 N/P ratio

The PEI content of the AMNPs was obtained by determining the weight loss from heating 20 mg of dehydrated PEI-coated AMNPs to 800 °C (at a rate of 20 °C/min) under air flow in a TA Instruments TGA Q500 thermo gravimetric analyzer. The PEI content was used to calculate the N/P ratio, the molar ratio of PEI nitrogen to pDNA phosphate. This N/P ratio is a measure of the ionic balance of the pDNA-PEI coated AMNP carriers. The positive charge is due to the nitrogen present in $NHCH_2CH_2$, which is the repeat unit of PEI with a molecular weight of 43 g/mol. The negative charge is due to the phosphate group present in the deoxyribose nucleotides. The average molecular weight of the nucleotides is 330 g/mol [200].

Example of N/P calculation: 0.5 µg pDNA was complexed with 0.125 µg of 25 kDa-PEI.

Phosphate molar concentration, $P = (0.5 \times 10^{-6})/330 = 1.52 \times 10^{-9}$ moles.

PEI molar concentration = $(0.125 \times 10^{-6})/(25 \times 10^3) = 5 \times 10^{-12}$ moles.

Number of repeating unit in 25 kDa-PEI = $(25 \times 10^3)/43 = 581$.

Nitrogen molar concentration, $N = 5 \times 10^{-12} \times 581 = 2.91 \times 10^{-9}$ moles.

Therefore, N/P ratio = $(2.91 \times 10^{-9})/(1.52 \times 10^{-9}) = 1.9$.

3.5 Transfection experiments

3.5.1 Cell culture

COS-7 monkey kidney cells (appendix A) were used in this work. These cells have been utilized extensively in research and this cell line is often an excellent choice for transfection experiments with recombinant plasmids [209]. They were cultured in Roswell Park Memorial Institute 1640 medium, supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin (complete RPMI) [210]. Cells were incubated at 37 °C in humidified 95% air and 5% CO₂ atmosphere, they were subcultured before they reach 90% confluency.

3.5.2 Preparation of gene vector complexes

Plasmids DNA (pDNA), pEGFP-N1 and pMIR-REPORT, were prepared as per protocol (appendix B). pEGFP-N1 codes for green fluorescent protein (GFP) and pMIR-REPORT contains a luciferase reporter gene. pMIR-REPORT was used in all the transfection studies, whereas pEGFP-N1 was used only in the plasmids comparison study. pDNA, polyMAG, Lipofectamine, PEI, uncoated and PEI-coated AMNPs were diluted in autoclaved distilled water. For the positive controls, optimized amount of 0.3 µg and 0.5 µg pDNA per well was complexed with 0.5 µl of lipofectamine and 0.5 µl of polyMAG respectively. For transfection using PEI and PEI-AMNP, 0.5 µg pDNA per well was used. The pDNA solutions were added to the gene vector solutions and thoroughly mixed by pipetting up and down 10 times. 25 µl of gene vector complexes per well were prepared in triplicates and the resulting complexes were incubated for 30 min at room temperature before use.

3.5.3 Magnetic and non-magnetic transfection

0.5×10^4 cells per well were seeded in 96-well plates one day before transfection and grown in complete Roswell Park Memorial Institute (RPMI) medium. At a confluency of about 50% to 60%, cells were washed with phosphate-buffered saline (PBS) and 125 μ l of incomplete RPMI (without FBS, L-glutamine and penicillin/streptomycin) per well was added.

25 μ l of pDNA/vector complexes were added into each well, making the total volume in each well equal to 150 μ l. For magnetically enhanced transfection, the 96-well plate was placed on top of a magnetoFACTOR plate for 20 min of incubation at room temperature. The magnetic field strength and magnetic field gradient used in this study were 0.13 T and 50 T/m. For non-magnetic transfection, incubation was done at room temperature for 20 min (5 h for PEI and Lipofectamine) under no magnetic field. The transfection medium was replaced with 200 μ l of complete RPMI after incubation. Incubation was continued for 24 h at 37 °C in humidified 95% air and 5% CO₂ atmosphere before the next medium change. In the incubation time study, incubation times of 5 min, 20 min, 40 min, 1 h, 3 h and 5 h were studied at 37 °C in humidified 95% air and 5% CO₂ atmosphere.

3.5.4 Static magnetic fields study

25 μ l of pDNA/PEI-AMNP complexes were added into each well, making the total volume in each well equal to 150 μ l. The 96-well plate was then placed on top of a magnetoFACTOR plate for 20 min of incubation at room temperature. The magnetic flux density and magnetic field gradient perpendicular to the well plate in the area of the cells depends on the separation between the cells and the magnetoFACTOR plate

(Table 3.2). The transfection medium was replaced with 200 μ l of complete RPMI after 20 min. The magnetoFACTOR plate was removed and incubation was continued for 24 h at 37 °C in humidified 95% air and 5% CO₂ atmosphere before the next medium change.

Table 3.2. Magnetic flux density and magnetic field gradient perpendicular to the well plate at different position above the magnet.

Position, z (mm)	Magnetic flux density (mT)	Magnetic field gradient (T/m)
2	250	80
4	130	50
8	35	11
∞	0	0

3.5.5 Pulsed magnetic fields study

Pulsed magnetic field transfection was performed using the same methodology as magnetic transfection under a static magnetic field, except that a pulsed magnetic field was applied for 5 min after subjecting the cells to a static magnetic field (130 mT) for 20 min. Different magnetic fields and frequencies perpendicular to the well plate were used in this study (Table 3.3). The pulsed field was generated using an electromagnet (PHYWE 06480.01) powered by a pulse power supply (Dynatronix DPR 40-30-100).

Table 3.3. Magnetic flux density and magnetic field gradient perpendicular to the well plate at different position above the electromagnet.

Magnetic flux density (mT)	Magnetic field gradient (T/m)	Frequency (Hz)
600	200	0, 10, 50, 100, 200, 500
30	10	50

3.5.6 Mechanism studies

To determine the mechanism of magnetic transfection, novel forms of magnetic transfection were performed, i.e., reverse magnetic transfection and retention magnetic transfection. Therefore, we denote the standard magnetic transfection as normal magnetic transfection. In normal magnetic transfection, cells were seeded 1 day before transfection. In reverse and retention magnetic transfection, pDNA/PEI-AMNP complexes were added and settled before adding the cells. In retention magnetic transfection, the magnetoFACTOR plate was placed beneath the 96-well plate until luciferase and viability assays were conducted (Figure 1.5). If endocytosis occurs, the transfection efficiency and viability of reverse magnetic transfection would be similar to that of normal magnetic transfection. Also, the transfection efficiency of retention magnetic transfection would be lower than that of reverse magnetic transfection as the magnetic field would have prevented the pDNA/PEI-AMNP complexes from being taken into the cells via endocytosis.

3.5.6.1 Normal magnetic transfection

0.5×10^4 cells per well were seeded in 96-well plates one day before transfection and grown in complete Roswell Park Memorial Institute (RPMI) medium. At a confluency of about 50% to 60%, cells were washed with phosphate-buffered saline (PBS) and 125 μ l of incomplete RPMI (without FBS, L-glutamine and penicillin/streptomycin) per well was added. 25 μ l of pDNA/PEI-AMNP complexes were added into each well, making the total volume in each well equal to 150 μ l. The 96-well plate was then placed on top of a magnetoFACTOR plate for 20 min of incubation at room temperature. The transfection medium was replaced with 200 μ l of complete RPMI after 20 min. The magnetoFACTOR plate was removed and

incubation was continued for 24 h at 37 °C in humidified 95% air and 5% CO₂ atmosphere before the next medium change.

3.5.6.2 Reverse magnetic transfection

In reverse magnetic transfection, pDNA/PEI-AMNP complexes were added and settled for 20 min before adding the cells. 25 µl of pDNA/PEI-AMNP complexes were added into each well containing 125 µl of incomplete RPMI. The 96-well plate was then placed on top of a magnetoFACTOR plate for 20 min of incubation at room temperature. The 150 µl of incomplete RPMI was replaced with 200 µl of complete RPMI containing 1×10^4 cells after 20 min. The magnetoFACTOR plate was removed and incubation was continued for 24 h at 37 °C in humidified 95% air and 5% CO₂ atmosphere before the next medium change.

3.5.6.3 Retention magnetic transfection

The methodology for retention magnetic transfection is the same as reverse magnetic transfection. The medium change occurred with the magnetoFACTOR plate in place. The magnetoFACTOR plate was placed beneath the 96-well plate until luciferase and viability assays were conducted.

3.5.6.4 Modeling and simulation

The aims of our model are to estimate the longest time taken for the DNA/PEI-AMNP complexes to settle on the cells and to determine whether the complex penetrates the cell during the 20 min of incubation under magnetic field.

The 2-D finite element model consists of 3 magnets to model the superimposing magnetic field present in the magnetoFACTOR plate and a well located at position z (Table 3.2) above the central magnet (Figure 3.1). In our analysis, the well has a radius of 3.2 mm and length of 6.4 mm. The magnet has a radius of 2.8 mm and a length of 5 mm and is magnetized to saturation, $M_s = 8 \times 10^5$ A/m ($B_r = 1$ T). The separation between the centres of the 2 magnets is 8.6 mm. The COMSOL Multiphysics software [211] was used for this analysis.

Figure 3.1. Geometric model used in COMSOL. The magnetization, M , of the central magnet is opposite to the magnets on the left and right.

At the cellular level, the cell is assumed to be a homogeneous Newtonian viscous fluid with a viscosity of 200 Ns/m^2 . The thickness of the cytosol at the centre of the nucleus, L_c , is $2 \text{ }\mu\text{m}$ (Figure 3.2). The DNA/PEI-AMNP complexes are assumed to be monodisperse and spherical. The effect of gravity is neglected due to the small size of the complex. Thus equation 2.2 becomes $\mathbf{F}_m + \mathbf{F}_f = 0$.

Figure 3.2. Cell model. The cell is assumed to be a homogeneous Newtonian viscous fluid.

Since $\mathbf{F}_f = -6\pi\eta R_{p,hyd}(\mathbf{v}_p - \mathbf{v}_f)$, $\mathbf{F}_f = \mathbf{F}_m$.

Velocity the DNA/PEI-AMNP complex, $\mathbf{v}_p$ is obtained as:

$$\mathbf{v}_p = \mathbf{F}_m / (6\pi\eta R_{p,hyd}) \quad (\text{Eq. 3.1})$$

The time taken for DNA/PEI-AMNP complexes to settle onto the cells, t_s , is:

$$t_s = L_m / \mathbf{v}_p \quad (\text{Eq. 3.2})$$

where L_m is the height of 150 μl of medium. The medium is non-magnetic ($\chi_f = 0$)

with a viscosity $\eta = 0.001 \text{ Ns/m}^2$ [159].

Similarly, the time for DNA/PEI-AMNP complex to traverse the cytosol, t_c , is:

$$t_c = L_c / \mathbf{v}_p \quad (\text{Eq. 3.3})$$

where L_c is the thickness of the cytosol at the centre of the nucleus and the viscosity of the cytosol is 200 Ns/m^2 [212].

3.5.6.5 Confocal laser scanning microscopy

50,000 cells per well were seeded 1 day before transfection in 6-well culture plates (TPP), each well contained a sterile glass coverslip. PEI-AMNPs were labeled with Oregon Green 488 carboxylic acid, succinimidyl ester as described in [202]. Rhodamine-labeling of pEGFP-N1 was performed with Label IT Tracker TM-Rhodamine Kit according with manufacturer's protocol. Transfection was performed using the same methodology as described in section 2.5.2.1, except that the amount of PEI-AMNPs and pDNA was 10 times more than that used in 96-well plate. After 20 min of incubation with and without magnetic field, each coverslip was washed twice with PBS. 1 ml of 4% formaldehyde was added to each well and incubated at 4 °C for 10 min. Each coverslip was subsequently repeatedly washed with PBS, and was lightly blot-dried by touching the coverslip on its edge on a tissue. The coverslip was gently placed inverted on a microscopic slide containing a small drop of mounting solution (VECTASHIELD hardset mounting medium with DAPI). Nail polish was used to seal the edges of the coverslip to the slide. Optical sectioning of the cells was conducted using a Carl Zeiss LSM 510 META upright microscope. Excitation of the green fluorophores was achieved using the 488 nm excitation line and detected as 524 nm wavelength. Red fluorophores were excited by 546 nm excitation line and detected as 576 nm wavelength. DAPI (4',6 diamidino-2-phenylindole) which stains the nucleus of the cell, produces blue fluorescence with excitation at about 360 nm and emission at about 460 nm. Optical sections were taken every 0.35 μm , the z resolution is 315 nm.

3.5.6.6 Cell wounding assays

Cell-impermeant tracer FITC-dextran

To determine whether the plasma membrane is disrupted during magnetic transfection, cell wounding and membrane repair assay was conducted [213, 214]. The wounded cell is recognized by the presence of the tracer FITC-dextran in the cytosol. Openings in the plasma membrane due to the wounding permit the tracer to enter and reseal, trapping the tracer inside the cell. RPMI was removed from the wells and 125 µl of FITC-dextran (0.5 mg/ml) per well was added to the cells. Magnetic transfection was subsequently performed as described in section 2.5.2. Cells were washed three times with incomplete RPMI. A fluorescence microscope (Olympus IX71) was used to detect wounded cells.

Cytosolic fluorescent marker Green Cell Tracker

Cell wounding could also be determined by the leakage of cytosolic material into the external medium [214]. Cells were incubated with 100 µl of Green Cell Tracker (10 µM) for 1 h at 37 °C in humidified 95% air and 5% CO₂ atmosphere. Cells were repeatedly washed with incomplete RPMI. Magnetic transfection was subsequently performed. A microplate reader (Tecan Infinite M200) was used to measure the fluorescence at an excitation wavelength of 485 nm and emission wavelength of 518 nm. Wells containing 150 µl of incomplete RPMI serve as negative controls and wells containing cells which were scratched 30 times with a syringe needle served as positive controls.

3.6 Transfection efficiency studies

After 48 h of post transfection incubation, determination of gene expression was performed. For GFP expression, the cells were washed with PBS and replaced with fresh complete medium. Transfected cells expressing GFP were detected with a

fluorescence microscope (Olympus IX71). Transfection efficiency was calculated as 100 times the number of green cells divided by the total number of cells. Ten fields were taken from the triplicates to determine the average.

For luciferase expression [215] the cells were washed with PBS and 20 µl of luciferase cell culture lysis reagent (CCLR) was added to each well. Cells were incubated for at least 15 min at room temperature for complete lysis. Luciferase assay reagent (100 µl for each assay) was kept at room temperature before performing luciferase assay. A luminometer (Turner Biosystems 20/20ⁿ) was programmed to perform a 10 s measurement read for luciferase activity. 20 µl of cell lysate was added to a luminometer tube containing 100 µl of luciferase assay reagent, and mixed by pipetting 5 times. Transfection efficiency (luciferase activity) was determined by light emission over 10 s, measured by the luminometer [216].

3.7 Cell viability

Cytotoxicity of the gene vectors was assessed in triplicates by crystal violet staining (CVS) assay [217] after 48 h post transfection. Cells were washed with PBS and 50 µl of 0.5% crystal violet solution in methanol was added to each well. Cells were incubated for 10 min at room temperature. The staining solution was discarded and the 96-well plate washed gently with tap water. The plate was placed upside down on paper towels to drain any remaining water. 100 µl of 1% SDS solution was added to each well to solubilize the stain. The plate was agitated until a uniform colour was obtained. Absorbance at 570 nm was measured by a microplate spectrophotometer (Bio-Rad Benchmark Plus). Cell viability (%) was computed as $(a/b) \times 100$, where a is OD₅₇₀ value derived from well containing pDNA/vector complexes and b is the mean

OD570 value derived from control wells (i.e., wells containing only cells and no pDNA/vector complexes).

3.8 Agarose Gel Electrophoresis

Complexes for this assay were prepared for N/P ratios of 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25 and 2.5. 1 μ L of diluted loading dye was added to each of the 50 μ L solutions containing 1 μ g of pDNA at the end of the 30 min incubation period. After brief vortex mixing, 20 μ L was retrieved from each resultant complex solution and loaded into the wells of 1 % agarose gel pre-stained with 1 mg/ml ethidium bromide. Samples were left to run at 100 V for 30 min in 1X TAE buffer and visualized under UV illumination with a UV transilluminator (UVP).

3.9 Statistical Analysis

All biological data are expressed as mean $\pm$ standard deviation. Luciferase activity, transfection efficiency and viability were analyzed by the single-factor ANOVA test. A p-value (p) less than 0.05 was considered to be significant.

CHAPTER 4: RESULTS

4.1 Outline

This chapter presents the characterization results obtained from TEM, XRD, VSM, TGA and photon correlation spectroscopy analysis. Transfection, confocal laser scanning microscopy and cell wounding assays results are also presented.

4.2 Characterization of MNPs

4.2.1 Individual MNP size

Figure 4.1 shows the TEM micrographs and size distribution of uncoated average sized 8 nm, 16 nm and 30 nm MNPs, the MNPs were predominantly equiaxed. The average particle size was calculated by determining the median size from a sample size of 75 to 165 particles.

Figure 4.1. TEM micrographs and size distribution plots of uncoated, average sized (A) 8 nm (B) 16 nm and (C) 30 nm MNPs. The MNPs were predominantly equiaxed. The average particle size was calculated by determining the median size from a sample size of 75 to 165 particles.

4.2.2 Phase of iron oxide

Figure 4.2 shows the XRD spectra of the uncoated 8 nm, 16 nm and 30 nm MNPs. The positions of the peaks and relative intensities indicate that the MNPs could be either magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$), hence Rietveld refinement analysis using TOPAS [218] was performed. Figure 4.3 shows a typical Rietveld refinement for 30 nm MNPs, it was found that both 8 nm and 30 nm MNPs were magnetite as the fit was better for magnetite compared to maghemite. For 16 nm MNPs, the fit was equally good for maghemite and magnetite (Figure 4.4), implying a higher volume fraction of maghemite phase in 16 nm MNPs compared to the 8 nm and 30 nm MNPs.

Figure 4.2. X-ray diffraction patterns of the uncoated 8 nm, 16 nm and 30 nm MNPs. The positions of the peaks and relative intensities indicate that the MNPs could be either magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$). Significant peak broadening was observed with decreasing particle size.

Figure 4.3. Rietveld refinement of 30 nm MNPs using (A) $\gamma\text{-Fe}_2\text{O}_3$ and (B) Fe_3O_4 as the possible crystal structures. The fit was better for magnetite compared to maghemite.

Figure 4.4. Rietveld refinements of 16 nm MNPs using (A) $\gamma\text{-Fe}_2\text{O}_3$ and (B) Fe_3O_4 as the crystallographic models. The fitting was equally good for maghemite and magnetite implying a higher volume fraction of maghemite phase in 16 nm MNPs compared to the 8 nm and 30 nm MNPs.

The crystallite size of the MNPs shown in Table 4.1 was determined using Scherrer formula [219]: $B_t = (0.9 \lambda) / (D \cos \theta)$, where D is the crystalline size, λ is the wavelength of the X-ray, θ is the Bragg angle and B_t is peak width (in radians) of the peaks. The calculated crystallite size from XRD (D_{ave}) agrees well with the particle size from TEM (D_{TEM}). Significant peak broadening was observed as the particle size decreases (Figure 4.2). The peak broadening effect can be explained by the Scherrer formula i.e., the crystalline size is inversely proportional to peak width, hence a decrease in particle size will cause the peak to broaden. Peak broadening could also

come from instrumental broadening and strain broadening. Instrumental broadening was corrected in the fundamental parameters function using [220] and further refined using standard materials from NIST (SRM 640a for silicon and SRM 660a for lanthanum hexaboride). Rietveld refinement was performed to obtain the strain and it was found the strains were 0.8 %, 0.5 % and 0.8 % in 8 nm MNP, 16 nm MNP and 30 nm MNP respectively.

Table 4.1. Calculated crystallite size for 8 nm MNP, 16 nm MNP and 30 nm MNP using Scherrer formula.

hkl	220	311	400	511	440	Diameter of uncoated MNP (by XRD), D _{ave} (nm)	Diameter of uncoated MNP (by TEM), D _{TEM} (nm)
Sample	Calculated crystallite size, D (nm)						
8 nm MNP	6.7	7.5	10.5	11.0	12.8	10	8 ± 2
16 nm MNP	16.2	18.6	18.9	24.1	26.4	21	16 ± 4
30 nm MNP	23.3	22.9	24.4	31.4	32.8	27	30 ± 10

4.2.3 Magnetic properties

Figure 4.5 shows the magnetization-field (M-H) loops of the uncoated MNPs measured at room temperature. 8 nm and 16 nm MNPs show superparamagnetic behavior, i.e. zero remanence. There is a small hysteresis loop for 30 nm MNPs due to the small volume fraction of particles larger than 30 nm possessing non-zero remanence. The Ms values for 8 nm, 16 nm and 30 nm MNPs were 43, 31 and 79 emu/g respectively, and were similar to the values reported by Kim [171] and Nishio [172]. The Ms value for 30 nm MNPs were close to those reported for bulk magnetite (82 emu/g) [221].

Figure 4.5. Magnetization-field (M-H) plots of uncoated 8 nm, 16 nm and 30 nm MNPs at room temperature. 8 nm and 16 nm MNPs exhibit superparamagnetic behaviour whereas there is a small hysteresis loop for 30 nm MNPs.

The M_s values of coated AMNPs were also high (27 to 75 emu/g), only 3 to 4 emu/g lower than the counterpart values of the uncoated particles. Susceptibility is defined by the relationship $M = \chi H$, where M is the magnetization of the material and H is the magnetic field strength. The susceptibility for coated AMNPs was comparable to values reported for uncoated AMNPs [171, 222-224]. The M_s and susceptibility (χ) values of PEI-AMNPs are tabulated in Table 4.2.

Table 4.2. Synthesis conditions and properties of 8 nm, 16 nm and 30 nm MNPs.

Sample	Volume ratio of NaNO₃: FeCl₂.4H₂O	Synthesis temperature (°C)	Aging conditions	Saturation magnetization of PEI-AMNPs, Ms (emu/g)	Volume susceptibility of PEI-AMNPs, χ	PEI content of PEI-AMNPs (wt %)
8 nm MNP	0:1	25	-	41 ± 1	1.9 ± 0.1	15
16 nm MNP	5:1	14	24 h, 4 °C	27 ± 1	1.5 ± 0.1	20
30 nm MNP	0:1	25	24 h, 4 °C	75 ± 2	8.1 ± 0.2	7

4.2.4 Hydrodynamic diameter and surface charge

The size distribution of the coated particles is shown in Figure 4.6. The hydrodynamic diameter and zeta potential of the magnetic transfection vectors and their respective complexes are presented in Table 4.3. The hydrodynamic diameters of DNA/PEI-AMNP complexes are in the range of 826 nm to 1378 nm, the zeta potentials of the complexes are about +16 mV.

Figure 4.6. Size distribution plots of coated (A) 8 nm (B) 16 nm and (C) 30 nm MNPs.

Table 4.3. Hydrodynamic diameters (nm) and zeta potentials (mV) of transfection vectors and their respective complexes.

Sample	Hydrodynamic diameters of vector (nm)	Zeta potential of vector (mV)	Hydrodynamic diameters of complex (nm)	Zeta potential of complex (mV)
PEI-A8MNP	625 ± 95	+ 18 ± 1	826 ± 86	+ 16 ± 1
PEI-A16MNP	759 ± 146	+ 19 ± 1	1378 ± 184	+ 16 ± 1
PEI-A30MNP	845 ± 88	+ 20 ± 1	962 ± 107	+ 17 ± 1

As described later, transfection efficiency and viability of the coated AMNPs were experimentally determined. The MNP/DNA ratio and N/P ratio for highest transfection efficiency and viability comparable to or better than polyMAG was determined, coated particles prepared using these MNP/DNA ratio and N/P ratio values are called “optimized” particles. Table 4.4 shows the values of the MNP/DNA ratio and N/P ratio of the optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP, the MNP/DNA ratios (w/w) varies from 0.9 to 1.5 and the corresponding N/P ratio varies in the range of 0.8 to 2.2. No particular trend of N/P ratio with increasing

particle size was observed. At the optimized ratio, there was complete complexation between the vector and DNA (Figure 4.7).

Table 4.4. MNP/DNA ratios and corresponding N/P ratios of PEI-AMNPs.

Sample	MNP/DNA ratio	N/P ratio
PEI-A8MNP	[#] 1.5	2.2
PEI-A16MNP	[#] 0.9	1.9
PEI-A30MNP	[#] 1.1	0.8
PEI-A8MNP	1.1	1.6
PEI-A16MNP	1.1	2.4

[#] optimized ratio

Figure 4.7. Agarose gel electrophoresis for (A) PEI, (B) PEI-A8MNP, (C) PEI-A16MNP and (D) PEI-A30MNP at N/P ratio from 0 to 2.5. Full complexation occurs at N/P ratio of 2.25, 0.75, 1 and 0.25 for PEI, PEI-A8MNP, PEI-A16MNP and PEI-A30MNP respectively.

4.3 Magnetically enhanced transfection

4.3.1 Optimization of PEI-A8MNP – luciferase activity assay and

CVS assay

Figure 4.9 shows a plot of the luciferase activity and viability for PEI-A8MNP as a function of MNP/DNA ratio in the range from 0 to 2.1 for a fixed incubation time of 20 min. The luciferase activity of naked DNA was close to the background level (cells only) and the luciferase activity for low MNP/DNA ratio (equal to 0.5) was comparable to that of naked DNA. With increased MNP/DNA ratio, luciferase

expression was enhanced. At MNP/DNA ratio equal to 1.1, luciferase activity increased sharply, reaching a maximum at MNP/DNA ratio equal to 1.5. Viability generally decreased with increasing MNP/DNA ratio. Interestingly, the transfection efficiency and viability for MNP/DNA ratios in the range of 1.1 to 2.1 were higher than the corresponding values for the polyMAG particles. A ratio of MNP/DNA equal to 1.5 was chosen as the optimum ratio for further investigation as it showed highest transfection efficiency as well as viability comparable to that of polyMAG.

Figure 4.8. Luciferase activity and viability for PEI-A8MNP as a function of MNP/DNA ratio for a fixed incubation time of 20 min. Transfection efficiency and viability for MNP/DNA ratios in the range of 1.1 to 2.1 were superior to the corresponding values of polyMAG particles. A ratio of MNP/DNA equal to 1.5 was chosen as optimum as it showed highest transfection efficiency and viability comparable to that of polyMAG. Luciferase activity and viability for optimum MNP/DNA ratio of PEI-A8MNP were significantly higher than PolyMAG, $P < 0.05$ vs. PolyMAG.

4.3.2 Effect of AMNPs

Figure 4.9 shows the luciferase activity and viability for PEI with N/P ratios in the range from 0 to 10, as well as for optimized PEI-A8MNP. The luciferase activity for low N/P ratio equal to 2 was comparable to that of naked DNA. Luciferase activity increased with increasing N/P ratio, with N/P ratio equal to 7.5 having similar transfection efficiency as optimized PEI-A8MNP. Viability decreased with increasing N/P ratio.

Figure 4.9. Luciferase activity and viability for PEI as a function of N/P ratio. Luciferase activity increased with increasing N/P ratio. Viability decreased with increasing N/P ratio. Optimized PEI-A8MNP with PEI content corresponding to a N/P ratio equal to 1.9 showed enhanced transfection compared to PEI with the same N/P ratio. Luciferase activities for PEI with ratios 2 and 5 were significantly lower than PEI-A8MNP, $P < 0.05$ vs. 8 nm PEI-AMNPs.

The incubation times for PEI-AMNPs in the presence of the applied magnetic field gradient were 20 min and 5 h for DNA and PEI. The amount of PEI on the optimized PEI-A8MNP corresponds to a N/P ratio of 2.2 (Table 4.3). The transfection efficiency

of the optimized PEI-A8MNP was 32 times that of pure PEI with N/P equal to 2. Significantly, only 20 min of incubation time was required, the significant increase in transfection efficiency, together with the much shorter incubation time confirmed the high efficacy of the magnetically enhanced transfection process.

4.3.3 Optimization of PEI-A16MNP and PEI-A30MNP

Optimization results for PEI-A16MNP and PEI-A30MNP are shown in Figures 4.9 and 4.10 respectively. Figure 4.10 shows the luciferase activity and viability for PEI-A16MNP as a function of MNP/DNA ratio in the range from 0 to 1.8 for a fixed incubation time of 20 min. Luciferase activity increased with increasing MNP/DNA ratio, reaching a maximum at MNP/DNA ratio equal to 0.9. Viability generally decreased with increasing MNP/DNA ratio, similar to the observations for PEI-A8MNP.

Figure 4.10. Luciferase activity and viability for PEI-A16MNP as a function of MNP/DNA ratio for a fixed incubation time of 20 min. Viability generally decreased with increasing MNP/DNA ratio. A ratio of MNP/DNA equal to 0.9 was chosen as optimum as it showed highest transfection efficiency and viability better than that of polyMAG. At this ratio luciferase activity was comparable to polyMAG, with viability 100% better than that of polyMAG. Luciferase activity for optimum MNP/DNA ratio of PEI-A16MNP was not significantly different from PolyMAG, $P > 0.05$ vs. PolyMAG.

Figure 4.11 shows the luciferase activity and viability for PEI-A30MNP as a function of MNP/DNA ratios in the range from 0 to 1.6 for a fixed incubation time of 20 min. Luciferase activity for low MNP/DNA ratio equal to 0.4 was comparable to that of naked DNA. At MNP/DNA ratio equal to 0.8, luciferase activity increased sharply, reaching a maximum at MNP/DNA ratio equal to 1.1. As with PEI-A8MNP and PEI-A16MNP, viability generally decreased with increasing MNP/DNA ratio. A ratio of MNP/DNA equal to 1.1 was chosen as optimum as it showed highest transfection efficiency and viability comparable to that of polyMAG. The luciferase activity of the optimum MNP/DNA ratio was higher than that of polyMAG.

Figure 4.11. Luciferase activity and viability for PEI-A30MNP as a function of MNP/DNA ratio for a fixed incubation time of 20 min. A ratio of MNP/DNA equal to 1.1 was chosen as optimum as it showed highest transfection efficiency and viability comparable to that of polyMAG. The luciferase activity and viability at the optimum MNP/DNA ratio were higher than that of polyMAG. Luciferase activity for optimum MNP/DNA ratio of PEI-A30MNP was significantly higher than PolyMAG, $P < 0.05$ vs. PolyMAG.

The optimized MNP/DNA ratios were found to be 0.9 and 1.1 for PEI-A16MNP and PEI-A30MNP respectively as compared to an optimum ratio of 1.5 for PEI-A8MNP; at these ratios, the highest transfection efficiency and viability comparable to or better than polyMAG were observed, both these sizes had comparable or better performance than polyMAG.

4.3.4 Particle size comparison study

Figure 4.12 shows the luciferase activity and viability of naked DNA, optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP, polyMAG and Lipofectamine. Magnetic vectors (PEI-AMNPs and polyMAG) under zero magnetic field served as additional

controls. The luciferase activity was normalized against that of Lipofectamine. Luciferase activities of magnetic vectors under magnetic field were four to seven times higher than that of magnetic vectors which were not subjected to a magnetic field. In the presence of a magnetic field, the transfection efficiencies of PEI-A8MNP and PEI-A30MNP were found to be 45% higher than polyMAG, whereas the transfection efficiencies of PEI-A16MNP and polyMAG were comparable. The transfection efficiencies of the PEI-A8MNP and PEI-A30MNP were ~80% that of lipofectamine. The viabilities of the PEI-AMNPs were about 2 times higher than that of lipofectamine, indicating that the PEI-AMNPs were less toxic than lipofectamine.

Figure 4.12. (A) Normalized luciferase activity (against Lipofectamine) and (B) viability for optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP for a fixed incubation time of 20 min. Magnetic vectors under no magnetic field served as additional controls. Luciferase activities of magnetic vectors under magnetic field were 4 to 7 times higher than that of magnetic vectors under no magnetic field. Under magnetic field, luciferase activities of PEI-A8MNP and PEI-A30MNP were 45% higher than polyMag and ~80% that of lipofectamine. Viabilities for the AMNPs under magnetic field were about 100% higher than lipofectamine. Luciferase activities for optimized PEI-A8MNP and PEI-A30MNP were significantly different from PolyMAG, $P < 0.05$ vs. PolyMAG.

4.3.5 Plasmids comparison study

Luciferase activity was determined from the pooled average of transfected and untransfected cells, and it reflects the relative transfection efficiency between different vectors. Whereas for GFP expression, transfection efficiency was obtained from the percentage of cells expressing the protein, thus reflecting the actual transfection

efficiency. The purpose of the plasmids comparison study was to determine whether the luciferase activity reflects the actual transfection efficiency. The reason why pEGFP-N1 was not used for all studies is the significant amount of time used in determining the GFP expression (counting the cells).

It was found that up to 36% of the cells were expressing GFP when lipofectamine was used. Figure 4.13 shows the transfection efficiency (normalized against Lipofectamine) and viability (normalized against control cells) of naked DNA, optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP, polyMAG and Lipofectamine using luciferase and GFP expressing plasmids. The transfection efficiencies for each vector using different plasmids were comparable, particularly for optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP. The viability was also similar in both plasmids. Generally, these results showed a similar trend as that seen in Figure 4.12, i.e. luciferase activity of naked DNA was close to the background level (cells only); luciferase activities of PEI-A8MNP and PEI-A30MNP were ~80% that of lipofectamine; viabilities for the PEI-AMNPs were about twice that of lipofectamine.

Figure 4.13. (A) Transfection efficiency and (B) viability of optimized PEI-A8MNP, PEI-A16MNP, PEI-A30MNP and polyMAG for a fixed incubation time of 20 min using luciferase and GFP expressing plasmids. The transfection efficiencies and viabilities of each vector for different plasmids were comparable ($P > 0.05$). This shows that luciferase activity directly reflects transfection efficiency.

4.3.6 Incubation Time Study

Figure 4.14 shows the luciferase activity of naked DNA, optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP, polyMAG and Lipofectamine for incubation times of 5 min, 20 min, 40 min, 1 h, 3 h and 5 h in the presence of a magnetic field gradient. Luciferase activity of lipofectamine increased with incubation time and reached a maximum after 5 h of incubation. Luciferase activities for PEI-AMNPs and polyMAG achieved near maximum activity after 20 min of incubation, except for PEI-A16MNP which required 40 min. Luciferase activities obtained from PEI-A8MNP, PEI-

A16MNP and PEI-A30MNP were higher than those of PolyMAG for incubation times of 1 h, 3 h and 5 h.

Figure 4.14. Luciferase activity of optimized PEI-A8MNP, PEI-A16MNP and PEI-A30MNP for varying incubation times ranging from 5 min to 5 h. Luciferase activity of lipofectamine increased with incubation time and reached a maximum after 5 h of incubation. Luciferase activities for PEI-AMNPs and polyMAG achieved near maximum activity after only 20 min of incubation, except for PEI-A16MNP which took 40 min. Luciferase activities of PEI-A8MNP, PEI-A16MNP and PEI-A30MNP were higher than PolyMAG ($P < 0.05$ vs. PolyMAG) at incubation times of 1 h, 3 h and 5 h.

Qualitative comparison of the amount of particles was performed after incubation. The medium was replaced with complete RPMI and the cells were observed under an optical microscope. Figure 4.15 shows the optical micrographs of untransfected COS-7 cells and cells transfected using PEI-A16MNP with incubation times of 20 min and 40 min. More particles were observed after 40 min of incubation (Figure 4.15C)

compared to 20 min of incubation (Figure 4.15B). For incubation times of 20 min and 40 min, similar quantity of particles was observed for the cases of PEI-A8MNP, PEI-A30MNP and polyMAG.

Figure 4.15. Optical micrographs of COS-7 cells. (A) Untransfected cells, (B) cells transfected using PEI-A16MNP with incubation times of 20 min and (C) cells transfected using PEI-A16MNP with incubation times of 40 min. There were more particles after 40 min of incubation than 20 min.

4.3.7 Effect of different static magnetic fields

The magnetic force experienced by the MNPs is dependent on the magnetic field intensity (Eq. 3.1). Figure 4.16 shows the luciferase activity and viability for PEI-A8MNP, PEI-A16MNP and PEI-A30MNP as a function of magnetic field strength. With no external magnetic field, the transfection efficiency was the lowest and viability the highest for all three sizes of PEI-AMNPs. Transfection efficiency increased with magnetic field, reaching a maximum at 130 mT and decreased thereafter. The lowest viability occurred at the highest magnetic field. Application of an optimum field of 130 mT resulted in a four to five-fold increase in transfection efficiency compared to cells which were not exposed to the magnetic field.

Figure 4.16. Luciferase activity and viability for (A) PEI-A8MNP, (B) PEI-A16MNP and (C) PEI-A30MNP as a function of magnetic field strength. With no external magnetic field, the transfection efficiency was the lowest and viability the highest for all three sizes of PEI-AMNPs. Transfection efficiency increased with magnetic field, reaching a maximum at 130 mT and decreased thereafter. The lowest viability occurred at the highest magnetic field. The luciferase activities for PEI-A8MNP and PEI-A30MNP under magnetic fields were significantly different ($P < 0.05$), whereas for PEI-A16MNP, the luciferase activities were comparable ($P > 0.5$).

4.3.7 Effect of additional pulsed magnetic field

Figure 4.17 shows the luciferase activity and viability for PEI-A8MNP subjected to 5 min of pulsed magnetic field after application of 20 min of optimum static magnetic field (130 mT). Frequencies in the range of 0-500 Hz were studied. No significant change in transfection efficiency or viability was observed due to the pulsed magnetic field.

Figure 4.17. Luciferase activity and viability for PEI-A8MNP under 5 min of pulsed magnetic field with frequencies in the range of 0-500 Hz after application of 20 min of static magnetic field (130 mT). There was no significant difference in either transfection efficiency or viability due to the presence of pulsed magnetic field ($P > 0.05$).

4.4 Mechanism studies

4.4.1 Comparison between normal, reverse and retention magnetic transfection

To determine the mechanism of magnetic transfection, comparison was made between normal, reverse and retention magnetic transfection. Figure 4.18 shows the luciferase activity and viability for different types of magnetic transfection under a magnetic field strength of 130 mT using PEI-A8MNP, PEI-A16MNP, PEI-A30MNP and polyMAG as the transfection vectors. Normal magnetic transfection yields the highest

transfection efficiency for all cases. The transfection efficiency for reverse and retention magnetic transfections were similar, in the range of 20-40 % of that of normal magnetic transfection. The transfection efficiency ratio of reverse to normal magnetic transfection for polyMAG was about half of that for the synthesized MNPs. Viability for reverse and retention magnetic transfections was 50-100 % higher than normal magnetic transfection.

Figure 4.18. Luciferase activity and viability for different types of magnetic transfection with PEI-A8MNP, PEI-A16MNP, PEI-A30MNP and polyMAG as the transfection vectors. Normal magnetic transfection yields the highest transfection efficiency for all cases. The transfection efficiency for reverse and retention magnetic transfections were similar, in the range of 20-40 % of that of normal magnetic transfection. Viability for reverse and retention magnetic transfection was 50-100 % higher than normal magnetic transfection.

To explain the difference in transfection efficiency between normal and reverse magnetic transfections, optical micrographs were taken. Figure 4.19 shows the typical optical micrographs of the DNA/PEI-AMNP complexes distribution under normal and reverse magnetic transfection. DNA/PEI-A8MNP complexes were observed in these

micrographs. The magnetic field used was 130 mT. Figure 4.19 also shows the distribution of DNA/PEI-AMNP complex aggregate size for normal and reverse magnetic transfection. DNA/PEI-AMNP complex aggregates were observed to cluster onto cells in normal magnetic transfection and uniformly distributed throughout the well in reverse magnetic transfection. Image analysis was performed using ImageJ software [225]. The average aggregate size for normal magnetic transfection ($7.3 \mu\text{m}^2$) was larger than that of reverse magnetic transfection ($3.7 \mu\text{m}^2$). The bigger DNA/PEI-AMNP complex aggregate size was due to the clustering effect on the cell.

Figure 4.19. Graphs showing the distribution of DNA/PEI-AMNP complex aggregate size and typical optical micrographs showing the DNA/PEI-AMNP complex distribution under normal and reverse magnetic transfection. The inset in the graph for normal magnetic transfection shows the size of the complex could be as large as 800 μm^2 . DNA/PEI-AMNP complex aggregates were observed to cluster onto cells in normal magnetic transfection and uniformly distributed throughout the well in reverse magnetic transfection. The DNA/PEI-AMNP complex aggregate size for normal magnetic transfection was larger than that of reverse magnetic transfection.

To establish why the transfection efficiency ratio of reverse to normal magnetic transfection for polyMAG was the lowest among the MNPs, transfection efficiency was determined 24 h after reverse and retention magnetic transfections. Figure 4.20

shows the micrographs of transfected cells 24 h after magnetic transfection using polyMAG for (A and B) reverse and (C and D) retention magnetic transfections. There were ten times more transfected cells for reverse magnetic transfection (37 %) compared to retention magnetic transfection (3.6 %).

Figure 4.20. Transmission and fluorescent micrographs showing transfected cells 24 h after magnetic transfection using polyMAG. (A and B) Reverse and (C and D) retention magnetic transfections. There were significantly more transfected cells in reverse magnetic transfection than in retention magnetic transfection.

4.4.2 Settling times for PEI-A8MNP, PEI-A16MNP and PEI-A30MNP under various static magnetic fields (0 - 250 mT)

Modeling was performed to estimate the longest times taken for the PEI-AMNPs to reach the cell surface (settling time) and the nucleus. Sample calculations of the magnetic force acting on the DNA/PEI-A8MNP complex at 130 mT, the

corresponding velocity of the complex and the time for the complex to reach the cell surface are as shown below:

From equation 2.4, magnetic force on the DNA/PEI-A8MNP complex,

$$\mathbf{F}_m = \frac{V_p(\chi_p - \chi_f)}{\mu_0} (\mathbf{B} \cdot \nabla) \mathbf{B} = 4 \times 10^{-14} \text{ N},$$

where $V_p = 4\pi r^3/3$, r = hydrodynamic radius of PEI-A8MNP = $(625 \times 10^{-9} \text{ m})/2$;

χ_p = magnetic susceptibilities of PEI-A8MNP = 1.9;

χ_f = magnetic susceptibilities of medium = 0;

μ_0 = magnetic permeability of free space = $1.26 \times 10^{-6} \text{ NA}^{-2}$;

$(\mathbf{B} \cdot \nabla) \mathbf{B}$ = magnetic force field = $0.213 \text{ kg}^2 \text{mA}^{-2} \text{s}^{-2}$ (obtained from finite element simulation).

From equation 3.1, velocity of the DNA/PEI-A8MNP complex,

$$\mathbf{v}_p = \mathbf{F}_m / (6\pi\eta R_{p,\text{hyd}}) = 5 \times 10^{-6} \text{ m/s},$$

where $\mathbf{F}_m = 4 \times 10^{-14} \text{ N}$;

η = viscosity of medium = 0.001 Ns/m^2 ;

$R_{p,\text{hyd}}$ = hydrodynamic radius of DNA/PEI-A8MNP complex = $(826 \times 10^{-9} \text{ m})/2$.

From equation 3.2, time taken for DNA/PEI-A8MNP complex to settle onto the cell,

$$t_s = L_m / \mathbf{v}_p = 1000 \text{ s} = 17 \text{ min},$$

where L_m = height of $150 \text{ }\mu\text{l}$ of medium = $5 \times 10^{-3} \text{ m}$;

$$\mathbf{v}_p = 5 \times 10^{-6} \text{ m}.$$

Figure 4.21 shows the calculated settling times for DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes as a function of magnetic field strength. Settling time decreases exponentially with magnetic field strength. Smaller MNPs need longer settling time. At magnetic fields greater than 130 mT, AMNPs with individual particle size larger than 8 nm in diameter would be captured within the magnetic transfection time of 20 min. All AMNPs with individual particle size greater than 30 nm would be captured within 20 min, regardless of the strength of the magnetic field.

Figure 4.21. Calculated settling times of DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes. Settling time decreases exponentially with magnetic field strength. Smaller MNPs need longer settling time. At magnetic fields greater than 130 mT, all the AMNPs with individual particle size bigger than 8 nm would be captured within the magnetic transfection time of 20 min. For large MNPs with individual particle size greater than 30 nm, all of them would be captured within 20 min regardless of the strength of the magnetic field.

To establish whether cell penetration occurs during magnetic transfection time of 20 min, computation was performed to determine the time taken for the DNA/PEI-AMNP

complex to reach the nucleus from the cell surface. Table 4.5 tabulates the gravitational force, magnetic forces (at the top of the medium and at the cell level) and time taken to the nucleus from the cell surface, t_c , for DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes when subjected to the maximum static magnetic field of 250 mT. The magnetic force experienced by the DNA/PEI-AMNP complex at the top of the medium can be up to two orders of magnitude stronger than the gravitational force. DNA/PEI-AMNP complex at the cell surface can experience a magnetic force up to 20 times stronger than at the top of the medium. The magnetic force also increases with MNP size.

Calculation for DNA/PEI-A8MNP complex under a pulsed magnetic field of 600 mT yields a magnetic force of ~5 pN, which is less than that experienced by DNA/PEI-A30MNP complex under a static magnetic field of 250 mT. The total estimated times required for DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes to reach the nucleus from the top of the medium under a magnetic field of 250 mT are 36 min, 14 min and 3 min respectively.

Table 4.5. Gravitational force, magnetic forces and time taken to traverse cytosol, t_c , for DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes when subjected to the maximum static magnetic field of 250 mT.

	DNA/PEI-A8MNP complex	DNA/PEI-A16MNP complex	DNA/PEI-A30MNP complex
Gravitational force (N)	6×10^{-15}	1×10^{-14}	2×10^{-14}
Magnetic force at the top of medium (N)	7×10^{-14}	2×10^{-13}	2×10^{-12}
Magnetic force at the cell level (N)	8×10^{-13}	3×10^{-12}	4×10^{-11}
t_c (min)	32	12	2

4.4.3 Confocal laser scanning microscopy

In this study, the normal magnetic transfection method was used. The effect of magnetic field on the distance traveled by DNA/PEI-A8MNP complex into the cell was tracked using confocal sectioning. Minimum and maximum static magnetic fields of 0 T and 250 mT respectively were examined. Figure 4.22 shows representative optical sections tracking of double-labeled DNA/PEI-A8MNP complexes after application of a magnetic field of (A) 0 T and (B) 250 mT for 20 min. Optical section at 0 μm was taken at the bottom of the cell. Optical section at 4.55 μm was taken near the top of the cell. The complexes (arrowed) were observed to extend 4.20 μm and 3.85 μm into the cell, without application of a magnetic field and magnetic field of 250 mT, respectively.

Figure 4.22. Tracking of double-labeled DNA/PEI-A8MNP complexes (indicated by an arrow) after 20 min of application of magnetic field of (A) 0 T and (B) 250 mT. Optical section at 4.55 μm was taken at the top of the cell, and optical section at 0 μm was taken at the bottom of the cell. The complex extended 4.20 μm into the nucleus in (A) and 3.85 μm in (B). Scale bar = 10 μm .

4.4.4 Wounding assays for cells subjected to maximum static and pulsed magnetic fields

Cell wounding assays were performed to ascertain if cells were wounded during magnetic transfection. Figures 4.23(A) and 4.23(B) show the result of the Green Cell Tracker cell wounding assay for maximum static magnetic field (250 mT) and maximum pulsed magnetic field (with frequencies in the range of 0-500 Hz), respectively. In both cases, there was no significant difference between samples and negative control (cells in iRPMI), indicating that cell wounding was absent.

Figure 4.23. Green Cell Tracker cell wounding assay for (A) maximum static magnetic field (250 mT) and (B) maximum pulsed magnetic field with frequencies in the range of 0-500 Hz. In both cases, there was no significant difference between samples and negative control (cells in iRPMI), indicating that cell wounding was absent.

Figure 4.24 shows the result of the FITC-dextran cell wounding assay. The sample was A8MNP and magnetic field strength used was 250 mT in normal magnetic transfection mode. Figures 4.24(A) and 4.24(B) show the bright field image and

fluorescence image of the positive control (scratch) respectively. Figures 4.24(C) and 4.24(D) show the typical bright field image and fluorescence image of the sample respectively. Wounded cells could be seen in the positive control (Figure 4.24(B)), indicating cell wounding. However, no wounded cells were detected in the other samples (Figure 4.24(D)), showing that cell wounding did not occur.

Figure 4.24. FITC-dextran cell wounding assay. (A) and (B) are bright field image and fluorescence image of positive control (scratch) respectively, (C) and (D) are typical bright field image and fluorescence image of sample. Green cells could be seen in the positive control (B), indicating signs of cell wounding. However, there were no green cells detected in all the other samples (D), thus signifying no cell wounding occurs.

CHAPTER 5: DISCUSSION

5.1 Outline

Our results show that magnetically enhanced transfection of COS-7 cells is feasible using the magnetic particles developed in this study. This study examined the effect of magnetic field strength and pulse frequency on transfection efficiency and viability, and also determined whether the magnetic complexes entered the cell via cell wounding due to the higher magnetic field strength and pulse frequency used in this study. Our results show that magnetofection of COS-7 cells does not proceed by cell wounding. We now discuss the specific results obtained in the context of previous work.

5.2 Characterization of MNPs

TEM and XRD analyses indicated that a narrow size distribution of iron oxide MNPs were obtained using the chemical synthesis techniques developed in this work. An average individual particle size of 8 nm, 16 nm and 30 nm were obtained. The saturation magnetization of these MNPs were comparable, for the corresponding size, to literature values [171, 172]. Lee *et al* reported that the saturation magnetization (Ms) value increases as the particle size increases [226]. Varanda *et al* also reported a linear relationship between Ms and particle size [207]. This was attributed to surface modification, e.g., defects at particle surfaces or disordered crystal orientation which could lead to the formation of non-magnetic layer, this factor is more important for smaller particles less than 10 nm diameter since they have high surface to volume ratio

and larger surface curvature. As a result, there is a significant decrease in the M_s value of 8 nm MNPs compared to the 30 nm MNPs (Table 1). However, in this study, the M_s value does not increase with particle size, e.g., the M_s value of 16 nm MNPs is lower than that of 8 nm MNPs. This could be explained by the presence of higher volume of maghemite phase in the 16 nm MNPs as indicated from Rietveld analysis refinement, this phase has a lower M_s value.

The M_s value represents the magnetic strength and susceptibility refers to the magnetic responsiveness to applied magnetic field. The magnetic strength of coated AMNPs were also high, only slightly lower than uncoated AMNPs (Table 4.2), hence the coated AMNPs were useful for efficient transfection. The 30 nm MNPs have the highest magnetic responsiveness, implying greater attraction to the external magnetic field. Both high magnetic strength and high magnetic responsiveness result in more MNPs being captured and settled on cells, hence higher transfection efficiency. It was observed that 16 nm MNP has higher PEI content than that of 8 nm MNP. This could be due to the lower pH of the suspension during PEI coating for 16 nm MNP. This would result in more PEI being protonated and thus more PEI being coated on 16 nm MNP. Another reason could be due to higher amount of negative charges on the surface of 16 nm MNP.

The optimized MNP/DNA ratios (w/w) range from 0.9 to 1.5, in general agreement with earlier reports that the optimized ratio was in the range of 1 to 3 depending on the particle type and gene vector [71]. The charge on the PEI-AMNPs can be explained as follows: Magnetite, being an amphoteric solid, develops charges in the protonation ($\text{Fe-OH} + \text{H}^+ \rightleftharpoons \text{Fe-OH}_2^+$) and deprotonation ($\text{Fe-OH} \rightleftharpoons \text{Fe-O}^- + \text{H}^+$) reactions of Fe-

OH sites on its surface [227], PEI molecules are subsequently attached on the surface of AMNPs by physisorption through Van der Waals forces [34, 74, 228]. After coating the AMNPs with PEI, the charges of the AMNPs were observed to be positive (Table 4.3) due to protonation of the NH_2 group, the pK_a of primary amines is around 5.5 [115]. After complexing with DNA, the hydrodynamic diameters of PEI-A8MNP and PEI-A30MNP increased by about 200 nm and 120 nm respectively, whereas the increase for PEI-A16MNP was about 620 nm. This increase could be attributed to the N/P ratio of the AMNP (Table 4.4), i.e., the higher the N/P ratio the bigger the increase in hydrodynamic diameter.

It is interesting to note that PEI-A30MNP has completely complexed with DNA at lower N/P ratio than PEI-A8MNP and PEI-A16MNP, even though PEI-A30MNP has lower PEI content than PEI-A8MNP and PEI-A16MNP. Higher PEI content should be completely complexed with DNA at lower N/P ratio. For this study in which the PEI is a shell surrounding the MNP core, the radius of the MNP core can affect the complexation behaviour (Figure 5.1). The PEI coating is thicker on A8MNP than on A30MNP. The thicker PEI coating on A8MNP enables shielding of the negative charges on the MNP surface, allowing negatively charged DNA to bond with PEI. This shielding effect is reduced for A30MNP due to the thinner PEI coating, lowering the amount of DNA that can bond with PEI-A30MNP.

Figure 5.1. Schematic diagram of PEI coating on A8MNP and A30MNP. The radius of the MNP core can affect the complexation behaviour. The PEI coating is thicker on A8MNP than on A30MNP. The thicker PEI coating on A8MNP enables shielding of the negative charges on the MNP surface, allowing negatively charged DNA to bond with PEI. This shielding effect is reduced for A30MNP due to the thinner PEI coating, lowering the amount of DNA that can bond with PEI-A30MNP.

5.3 Effectiveness of magnetically enhanced transfection

Transfection efficiency was increased ~32 times by the use of PEI-AMNPs under a magnetic field compared to PEI with equivalent N/P ratio (Figure 4.9). Transfection efficiency of magnetic vectors under magnetic field was four to seven times higher than that of magnetic vectors under no magnetic field (Figure 4.12). This enhancement was attributed to the physical concentration of DNA at the cell surface. Luo and Saltzman showed that the low DNA concentration at the cell surface was the barrier that limits transfection efficiency [229]. They used silica nanoparticles, which by themselves do not deliver DNA, to enhance DNA delivery mediated by other transfection reagents; the increased concentration of DNA was achieved by settling of DNA/nanoparticle complexes under gravity. In our case, the increased concentration

of DNA at the cell surface was achieved by the magnetic force on the DNA/PEI-AMNP complexes which pulls them towards the cells.

Significantly, the MNPs also reduced the incubation time from 5 h to 20 min, it also considerably decreased the amount of PEI required for transfection. PEI with N/P ratio equal to 7.5 was needed to achieve transfection efficiency similar to that of PEI-AMNPs having N/P ratio equal to 2.2 (Figure 4.9).

5.4 Effect of PEI-AMNP properties on transfection efficiency

We fixed the MNP/DNA ratio equal to 1.1, this value is the optimized value for PEI-A30MNP. This value was also used for PEI-A8MNP and PEI-A16MNP. Figure 5.2 shows plots of normalized transfection efficiency as a function of physical properties (A) M_s , (B) χ , (C) N/P ratio, (D) zeta potential and (E) hydrodynamic diameter of the complex at MNP/DNA ratio equal to 1.1 for a fixed incubation time of 20 min. The transfection efficiency of PEI-A8MNP, PEI-A16MNP and PEI-A30MNP with MNP/DNA ratio equal to 1.1 were normalized against the transfection efficiency of Lipofectamine. The data for PEI-A16MNP was interpolated from the values of MNP/DNA ratio of 0.9 and 1.3 in Figure 4.10 and normalized against the transfection efficiency of Lipofectamine. Linear regression was performed to determine the correlation between transfection efficiency and each physical property. The transfection efficiency is strongly positively correlated to M_s and χ , strongly negatively correlated with N/P ratio, moderately positively correlated to zeta potential and moderately negatively correlated to hydrodynamic diameter of the complex.

Figure 5.2. Transfection efficiency of PEI-A8MNP, PEI-A16MNP and PEI-A30MNP as a function of (A) Ms, (B) χ , (C) N/P ratio, (D) zeta potential and (E) hydrodynamic diameter at MNP/DNA ratio equal to 1.1 for a fixed incubation time of 20 min. Transfection efficiency was normalized against that of Lipofectamine. The transfection efficiency is strongly positively correlated to Ms and χ , strongly negatively correlated to N/P ratio, moderately positively correlated to zeta potential and moderately negatively correlated to hydrodynamic diameter.

A strong linear trend between transfection efficiency and Ms (R^2 of 1), χ (R^2 of 0.92), and N/P ratio (R^2 of 0.93), and a moderate linear trend for zeta potential (R^2 of 0.79)

and hydrodynamic diameter (R^2 of 0.35) was observed. PEI-AMNPs with better magnetic properties have higher transfection efficiency since they can be easily captured by an external magnetic field, resulting in shorter magnetically enhanced transfection time as well as higher transfection efficiency since more DNA is concentrated at the cell surface. However, it should be noted that high Ms also results in larger agglomerates due to increased attractive forces between particles, these larger aggregates may be too large to be taken up by the cell.

Higher N/P ratio results in lower transfection efficiency. PEI-A16MNP has the highest N/P ratio, i.e., a larger amount of PEI, thus more protons can be absorbed to cause both endosome destabilization (“proton sponge” effect) as well as to provide a larger physical barrier to degradative enzymes. However, a larger amount of PEI could translate to higher cell death due to PEI toxicity [230]. Higher N/P ratio also means larger amount of positive charges from PEI. The surface charge plays an important role in the formation of complexes and association with the cell membrane [192]. Sufficient positive surface charge is also critical for DNA compaction, which serves to protect the DNA [38]. However, too high a charge implies that DNA will not be released from the complexes easily and fewer cells were be transfected.

Large complex results in lower transfection efficiency. From literature, there is no universal optimum hydrodynamic diameter as cells vary in size and structure. Chorny *et al* transfected rat aortic smooth muscle cells and bovine aortic endothelial cells and reported that 375 nm sized polylactide AMNP exhibited higher transfection rates compared with 185 nm and 240 nm sized AMNP [31]. Thorek and Tsourkas [231] studied the cellular uptake of superparamagnetic iron oxide (SPIO) in non-phagocytic

T cells over a range of particle sizes ranging from 33 nm to 1.5 μm . They found efficient labeling of cells for particle sizes up to 300 nm, micron-sized particle uptake was limited. The low transfection efficiency of PEI-A16MNP could be due to limited particle uptake as its complex size is in the micron range.

Foged *et al* investigated the uptake of polystyrene particles by dendritic cells in a broad particle size range (0.04 μm to 15 μm) and variable surface properties, they reported that uptake of larger particles could be greatly enhanced with a positive surface charge [232]. In the case of magnetic transfection, the dependence on a positively charge surface is less critical because the magnetic force will pull the magnetic complex towards the cell surface regardless of the charges on complex surface. In the present study, the correlation between zeta potential and transfection efficiency is statistically insignificant as the difference in zeta potentials were only 1 mV.

5.5 Plasmids comparison

The normalized transfection efficiencies (against Lipofectamine) were similar for each vector (Figure 4.13), indicating that the number of plasmids entering the cell nucleus was similar for luciferase and GFP plasmids. Thus the luciferase activity reflects the transfection efficiency, hence, the term “transfection efficiency” is used interchangeably with “luciferase activity”.

While viability is significantly higher in magnetically enhanced transfection, transfection efficiency is lower. This indicates that the amount of DNA taken up by

each cell is lower, leading to a reduced signal per cell (based on data using GFP plasmid in Fig. 4.12). Our data indicates that a key limitation of magnetically enhanced transfection is the amount of DNA that gets into cells - if this can be improved it may be possible to get even higher transfection efficiencies without decreased viability. Also, increased viability would indicate that the cells are less stressed during magnetically enhanced transfection. This would be advantageous in applications where higher cell viability is required. Although the transfection efficiencies of PEI-A8MNP and PEI-A30MNP are lower than that of Lipofectamine, the ability to actively target tissues and its higher cell viability render magnetic transfection a more versatile technique than lipofection for *in vivo* applications.

5.6 Incubation time study

Our results show that Lipofectamine requires at least 3 h of incubation to achieve near maximum transfection efficiency. For PEI-AMNPs including polyMAG, near maximum transfection efficiency was achieved after only 20 min of incubation, except for PEI-A16MNP which required 40 min. This is in agreement with the recommended incubation time of 20 min stated in the protocol for polyMAG [68]. Although Lipofectamine was observed to perform as well as PEI-A8MNP and PEI-A30MNP with 20 min of incubation time, it is important to note that this is not necessarily the case in other cell types, especially primary cells [59, 68, 233]. More importantly, Lipofectamine would not be as effective as magnetic nanoparticles for *in vivo* gene delivery because Lipofectamine lacks the ability to target tissues. Increasing the dose of Lipofectamine to achieve better *in vivo* transfection efficiency would result in acute inflammation and tissue damage [234].

For PEI-A16MNP, there was a much larger difference in transfection efficiency between 20 min and 40 min of incubation as compared to PEI-A8MNP, PEI-A30MNP and polyMAG. This could be explained by the significant difference in the amount of particles captured by the magnetic field gradient after 20 min and 40 min of incubation for PEI-A16MNP (Figure 4.15). For PEI-A8MNP, PEI-A30MNP and polyMAG, there was little difference in the amount of particles. This difference was attributed to the weaker magnetic properties of PEI-A16MNP (Table 4.2) compared to PEI-A8MNP, PEI-A30MNP or polyMAG particles, a longer time was therefore required to capture a similar amount of particles. This further emphasizes the importance of magnetic properties in the magnetically enhanced transfection process.

5.7 Effect of different static magnetic fields on transfection efficiency

For the MNPs sizes studied, transfection efficiency initially increased with magnetic field, reaching a maximum at 130 mT. Since the MNPs consist of PEI which is toxic at high concentration, increased internalization means more cell death, hence viability decreased with increasing magnetic field. Hence, higher cell death at 250 mT resulted in lower overall transfection efficiency, these results suggest that there is an optimum magnetic field to achieve maximum transfection efficiency. Previous studies [72, 162] have suggested that higher transfection efficiency was due to increased concentration of magnetic complexes on the cell surface. Thus, to increase the concentration of magnetic complexes, one could either use a higher magnetic field or a longer incubation time. In this work, the optimum magnetic field is 130 mT for an incubation time of 20 min. Generally, the magnetic field reported in literature [17, 30, 53, 72,

160, 162] is 250 mT for an incubation between 5 to 15 min. It is therefore recommended to optimize either the magnetic field or incubation time to achieve the maximum transfection efficiency

5.8 Effect of pulsed magnetic field on transfection efficiency

Interestingly, our results did not show statistically significant difference in transfection efficiency and viability due to the pulsed magnetic field, even with a magnetic field strength of 600 mT. This magnetic field is ~22 times stronger than that used by Kamau et al [30, 160]. They used a magnetic field of 27 mT with frequencies of 50 Hz and 0.75 Hz in the vertical and horizontal directions respectively. They reported a two-fold increase in transfection efficiency over cells exposed only to the static field.

The Neel relaxation time calculated using equation 2.1 where $K = 40 \text{ kJ/m}^3$ [206], $\tau_0 = 10^{-9} \text{ s}$ [205, 235] and $T = 300 \text{ K}$, gives a value of 13 ns for 8 nm MNP. This is much shorter than the experimental time of 2 ms for a frequency of 500 Hz. This means the particles will behave paramagnetically and the ‘drilling’ mechanism should still be in effect. Also, the magnetic susceptibility at 500 Hz is the same as in a static field, thus the magnetic force experienced by the particle is similar.

In our work, we have exposed the cells to vertical oscillations, due to equipment limitation no horizontal pulsed field was applied. Thus the difference between ours and Kamau’s results could be due to the absence of horizontal oscillations in our study. Dobson has suggested that by introducing an oscillating, horizontal component

of motion to the particles, energy would be added to the transfection system [76]. They reported studies in HEK293 cells and H292 human lung epithelial cells which showed a ten-fold increase in transfection level in comparison to static field. They suggested that horizontal motion may prove useful for promoting penetration of the mucous lining in the lung and enable delivery of therapeutic genes for cystic fibrosis. Also, gene transfer occurred within 5 min of exposure to pulsed magnetic field, this rapid uptake of nucleic acids before systemic clearance occurs is particularly useful for *in vivo* applications [76].

5.9 Mechanism of magnetically enhanced transfection

To gain insight into the mechanism of magnetic transfection, modified forms of the standard magnetic transfection protocol were performed, namely reverse and retention magnetic transfection. Modeling, confocal laser scanning microscopy and cell wounding assays were also performed. The transfection efficiency for reverse magnetic transfection was 20 – 40 % that of normal magnetic transfection and the viability for reverse magnetic transfection is 30 – 40 % higher than normal magnetic transfection (Figure 4.18). It was observed that MNPs clustered onto cells in normal magnetic transfection whereas MNPs were uniformly distributed in reverse magnetic transfection (Figure 4.19). The difference in distribution of magnetic particles between normal and reverse magnetic transfection was due to the interaction between cells and magnetic particles. In the normal method, the magnetic particles were added after the cells. The plasma membrane of the cells are negatively charged, which attracts the magnetic particles containing positively charged PEI. In the reverse method, the magnetic particles were dispersed in the absence of cells, resulting in an uniform distribution of particles. The clustering of MNPs onto cells means that the

concentration of MNPs per cell is higher in normal magnetic transfection than in reverse magnetic transfection. This observation is in agreement with previous studies [72, 162], which suggest that higher transfection efficiency was due to increased concentration of magnetic complexes on the cell surface. Having higher concentration of magnetic complexes could also interfere with the functioning of the plasma membrane and hence result in cell death. Another reason for the higher transfection efficiency of normal magnetic transfection could be that normal magnetic transfection occurs by cell wounding, whereas endocytosis is the operating mechanism in reverse magnetic transfection.

The transfection efficiency and viability of reverse and retention magnetic transfection were similar, indicating that similar amount of DNA entered the nucleus after 48 h. This suggests a similar mechanism occurring in reverse and retention magnetofection, it is likely that for retention magnetofection, DNA or DNA/PEI is detached from the magnetic complex and entered the cell through endocytosis, resulting in similar transfection efficiency as reverse magnetic transfection.

Interestingly for polyMAG, the number of transfected cells after 24 h for retention magnetic transfection was ten times lower than reverse magnetic transfection (Figure 4.20). Also, the transfection efficiency ratio of reverse to normal magnetic transfection for polyMAG is observed to be about half of that for the synthesized PEI-AMNPs. Both observations suggest that DNA was more strongly bound to polyMAG than the synthesized PEI-AMNPs and could explain why polyMAG has the lowest transfection efficiency among the MNPs studied in this work.

The objectives of our model are to estimate the longest time taken for the DNA/PEI-AMNP complexes to settle on the cells and to determine whether the complex penetrates the cell during the 20 min of incubation under magnetic field. The modeling results show that at a magnetic field of 130 mT, all the DNA/PEI-AMNP complexes were captured within the incubation time of 20 min. This result was based on the assumption that the DNA/PEI-AMNP complexes were mono-disperse, however, experimental data indicates they were poly-disperse [236]. Thus, not all the DNA/PEI-AMNP complexes would be captured within 20 min, especially the smaller size DNA/PEI-AMNP complexes. Applying a higher magnetic field of 250 mT would capture more DNA/PEI-AMNP complexes within 20 min and increase the concentration of DNA/PEI-AMNP complexes on the cell surface. This could lead to increased toxicity to the cells, as indicated by the decreased viability (Figure 4.16).

It was calculated that under a pulsed magnetic field of 600 mT, DNA/PEI-A8MNP complex experiences a magnetic force of 5 pN. This is much less than the 40 pN experienced by DNA/PEI-A30MNP complex under a static magnetic field of 250 mT (Table 4.5). (It is noted here that the calculation of the magnetic force reported by Furlani [159] overestimates the force by a factor of 1000 [237].) This shows that a much higher magnetic field is required to wound the cell. An alternative method can be to use MNPs with higher saturation magnetization.

The longest total predicted times required for DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes to reach the nucleus from the top of the medium under a magnetic field strength of 250 mT are 39 min, 15 min and 3 min respectively

(Table 4.5). These results suggest that cell wounding should occur within the incubation time of 20 min.

Comparison of the distance the DNA/PEI-AMNP complex travels into the cells with (250 mT) and without magnetic field was performed via confocal sectioning. It was hypothesized that if cell wounding was to occur, the complex subjected to a magnetic field of 250 mT would travel further into the cell than the complex under no magnetic field. Interestingly, our results show that the complex under no magnetic field extended further into the cell (4.20 μm) than the complex under a magnetic field of 250 mT (3.85 μm). As the z resolution is 315 nm, both complexes were considered to travel about the same distance into the cell, suggesting that cell wounding did not occur when subjected to a magnetic field of 250 mT.

Results from both cell wounding assays show that cells were not wounded even when they were subjected to the maximum static (250 mT) and pulsed (600 mT) magnetic fields. This suggests that the same mechanism occurs for both static and pulsed fields. Previous studies of the mechanism of magnetofection using similar magnetic field strength [72, 162] also concluded that cellular uptake of magnetic complexes proceeds by endocytosis.

CHAPTER 6: CONCLUSIONS AND FUTURE WORK

6.1 Outline

This chapter presents the significant findings from this work and recommendations for future studies.

6.2 Conclusions

Gene therapy is an exciting development in medical treatment because it aims to treat the cause of diseases rather than the symptoms. Serious adverse consequences have resulted from the use of viral gene delivery systems, hence, significant amount of efforts have been focused on improving non-viral gene delivery systems. Magnetic nanoparticle assisted gene delivery is a promising technique with its rapid and efficient delivery of nucleic acids to the target cells. However, there is insufficient research on aspects related to the magnetic carriers and the mechanism of magnetic transfection. In this work, one of the major contributions is the correlation between the transfection efficiency and the physical parameters of the magnetic nanoparticles. PEI-AMNPs were synthesized and characterized using TEM, XRD, VSM, TGA and PCS techniques. Optimization of the transfection efficiency of these particles was performed by *in vitro* magnetic transfection studies using COS-7 cells. The effects of magnetic field strength, and static and pulsed magnetic fields on the transfection efficiency and cell viability were also examined. Another major contribution is the use of novel forms of magnetic transfection, modeling, CLSM and cell wounding assays to study the mechanism of magnetic transfection. The following are the conclusions and implications drawn from this work:

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- Iron oxide MNPs of average individual particle diameter of 8 nm, 16 nm and 30 nm were synthesized by the co-precipitation technique. 8 nm and 30 nm MNPs were magnetite and 16 nm MNPs were maghemite, as determined by XRD and Rietveld refinement analysis. This explains why the saturation magnetization of 16 nm MNPs is lower than that of 8 nm MNPs. It is important MNPs have high saturation magnetization as it will result in more MNPs being captured and settled on cells, and hence higher transfection efficiency.
 - 8 nm and 16 nm MNPs exhibit superparamagnetic behaviour, whereas 30 nm does not. The saturation magnetization values of PEI coated MNPs were only slightly lower than those of the uncoated MNPs, demonstrating that the PEI coating did not significantly affect the magnetic properties. Thus, these particles were still useful for efficient magnetic transfection.
 - In the presence of MNPs under a magnetic field, transfection efficiency was increased ~32 times compared to PEI with equivalent N/P ratio. Transfection efficiency of MNPs under a magnetic field was four to seven times higher than that of MNPs not subjected to a magnetic field. To achieve similar level of transfection, considerably small quantity of PEI was required. PEI with a N/P ratio of 7.5 was needed to achieve transfection efficiency similar to that of MNPs coated with PEI having N/P ratio equal to 2.2. The use of MNPs also dramatically reduced incubation time from 5 h to 20 min. The significant increase in transfection efficiency, smaller quantity of PEI required and much

shorter incubation time demonstrate the speed and efficiency of magnetic transfection.

- Transfection efficiency was strongly positively correlated with magnetic properties (M_s and χ), strongly negatively correlated with N/P ratio and moderately negatively correlated to hydrodynamic diameter of the complex. This means that MNPs with higher transfection efficiency have superior M_s and χ , lower N/P ratio and smaller complex size. MNPs with weaker magnetic properties require longer incubation time to achieve similar level of transfection as MNPs with superior magnetic properties.
- An optimum magnetic field exists, for PEI-coated 8 nm and 30 nm AMNPs, the magnetic field required for maximum transfection efficiency is 130 mT with incubation time of 20 min. Under these conditions, their transfection efficiencies were ~45% higher than polyMAG, a commercial magnetic formulation. This is an improved performance.
- Additional pulsed magnetic field strength of 600 mT with frequency ranging from 0 to 500 Hz has no effect on transfection efficiency and viability, contrary to previous reports. This could indicate the importance of the horizontal component of the pulsed magnetic field.
- Through the use of novel modified forms of magnetic transfection (reverse and retention) together with normal magnetic transfection, it was found that higher

concentration of complexes per cell resulted in higher transfection efficiency and lower viability.

- Modeling results showed that at magnetic field strength of 130 mT, all the DNA/PEI-A8MNP, DNA/PEI-A16MNP and DNA/PEI-A30MNP complexes would be captured within the incubation time of 20 min. Also, under a magnetic field strength of 250 mT, cell wounding would have occurred during the 20 min of incubation period if it was the operating mechanism. These results provide the theoretical basis for the mechanism studies using CLSM and cell wounding assays.
- For the first time, mechanism studies using a combination of modified forms of magnetic transfection, CLSM and cell wounding assays were used in the context of magnetic transfection to show that cell wounding is absent in magnetic transfection.

In summary, this work demonstrated that magnetic nanoparticle assisted gene delivery did enhance transfection by increasing the concentration of complexes on the COS-7 cell surface. Optimization of physical parameters and magnetic field strength is required to achieve high transfection efficiency and good viability. The physical parameters that will significantly determine the overall transfection efficiency of the MNPs for gene delivery are the magnetic properties, N/P ratio and complex size. The effect of zeta potential is inconclusive as the zeta potentials were very similar among the various complexes. The mechanism of magnetic transfection is endocytosis and not through cell wounding.

6.3 Future work

The present work has explored limited but important aspects of magnetic nanoparticle assisted gene delivery. Using this work as a foundation, the following further studies can be performed:

- The size of the complex in the present work range from around 800 nm to 1500 nm. Smaller complex size can be studied to gain a better insight into the effect of size on transfection efficiency and viability.
- The effect of zeta potential can be explored because in the present study the zeta potentials were very similar among the various complexes.
- In this work, transfection studies were carried out in COS-7 cells. The same set of transfection work can be done on other commonly used cells such as HeLa, 293T and CHO, and also on non-permissive cells such as NIH3T3 and primary cells to determine whether the results obtained in this work apply to other cell types.
- Pulsed magnetic field did not seem to have any effect on transfection efficiency and viability in this work. This could be due to the absence of horizontal component of the pulsed magnetic field. The device used by Kamau et al [30] could be utilised and modified to have higher frequencies. Alternatively, a more powerful electromagnet which can generate higher magnetic field strengths can be used to determine the point where cell wounding takes place.
- For modeling work, our simulation results showed that cell wounding should have occurred but it was not in experiments. Hence, a more accurate cell model that gives a better representation of the cell membrane should be used.

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PUBLICATIONS AND CONFERENCES

Journal Publications

1. **D. Ang**, Q.V. Nguyen, S. Kayal, P.R. Preiser, R.S. Rawat and R.V. Ramanujan, “Insights into the mechanism of magnetic particle assisted gene delivery”, **Acta Biomaterialia**, 7, 1319-1326 (2011).
2. **D. Ang**, C.Y. Tay, L.P. Tan, P.R. Preiser and R.V. Ramanujan. “*In vitro* studies of magnetically enhanced transfection in COS-7 cells”, **Materials Science and Engineering C**, 31, 1445-1457 (2011).

Conference Presentations

1. **D. Ang**, P.R. Preiser and R.V. Ramanujan, “Functionalized Magnetic Nanoparticles for Therapeutic Gene Delivery”, in International Conference on Materials for Advanced Technologies (ICMAT), 2011 (accepted).
2. **D. Ang**, P.R. Preiser and R.V. Ramanujan, “Magnetofection: *In Vitro* Studies of Magnetic Particle Assisted Gene Delivery”, in International Conference on Materials for Advanced Technologies (ICMAT), 2009.

APPENDIX

A: COS-7 cell line [209, 238]

The COS-7 cell line was derived by Yakov Gluzman in the early 1980s from the previously established CV-1 African green monkey kidney line by transformation of the normal cells with an origin defective mutant of simian virus 40 (SV40) that codes for the wild-type virus T-antigen. The fibroblast line (Figure A-1) adheres to glass and plastic in culture and is generally utilized as a transfection host.

COS-7 cells are susceptible to SV40 (tsA209 strain) at 40 °C, and SV40 mutants with deletions in certain regions. Because of this susceptibility, COS-7 cells have been utilized extensively in research, and the cell line is often an excellent choice for transfection experiments with recombinant plasmids. In addition, SV40 itself is a popular subject of study largely due to the fact that the small DNA tumor virus features a relatively simple genetic organization and a genome that can be manipulated with relative ease. Moreover, SV40 has been of exceptional scientific interest due to its possible association with certain types of human cancers, especially mesothelioma.

Enclosed in a spherical capsid, SV40 DNA is 5243 nucleotides in size and exhibits a closed circular superhelical conformation. The capsid that encloses the supercoiled DNA is so small that there is only enough space for the genome to encode a small number of functions. The lifecycle of the virus is controlled by a regulatory portion of the genome, which also encodes three capsid proteins. Space is so limited in the genome that the capsid proteins are encoded with reading frames that overlap, so that

the final nucleotide sequence of the gene for one protein also encodes for the initial portion of another protein. In addition to capsid proteins, SV40 DNA encodes the potentially tumor-causing protein T-antigen and a spliced version of the protein called small t-antigen. In primate cells, T-antigen usually controls SV40 DNA replication as well as the packaging of the virus into capsids, but in many other cells the protein binds to p53 and Rb proteins, which are important in the regulation of growth, blocking their normal processes and resulting in the development of tumors.

Figure A-1. Optical micrograph of COS-7 cells [209].

B: Protocol for pMIR-REPORT and pEGFP plasmids

preparation

The following protocol is for plasmid DNA purification using QIAGEN Endofree Plasmid Maxi Kit [239].

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 3 ml LB medium containing the appropriate selective antibiotic. Incubate for 6 h at 37 °C with vigorous shaking (200 rpm). (Use a tube or flask with a volume of at least 4 times the volume of the culture.)
2. Dilute the starter culture into 1L conical flask containing 250 ml Amplicin or Kanamycin (Amplicin for pVLH and Kanamycin for pEGFP) LB medium. Grow at 37 °C for 12-16 h with vigorous shaking (200 rpm).
3. Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4 °C. (Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.)
4. Resuspend the bacterial pellet in 10 ml Buffer P1. (The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.)
5. Add 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4-6 times, and incubate at room temperature (15-25 °C) for 5 min. (Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow this lysis reaction to proceed for more than 5 min. During the incubation prepare the QIAfilter Cartridge: Screw the cap onto the outlet nozzle of the QIAfilter Maxi Cartridge. Place the QIAfilter Cartridge in a convenient tube.)

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6. Add 10 ml chilled Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4-6 times. Proceed directly to step 7. Do not incubate the lysate on ice. (Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and KDS becomes visible. The buffers must be mixed completely. If the mixture still appears viscous, more mixing is required to completely neutralize the solution. It is important to transfer the lysate into the QIAfilter Cartridge immediately in order to prevent later disruption of the precipitate layer.)
 7. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature (15-25 °C) for 10 min. Do not insert the plunger. (Important: This 10 min incubation at room temperature is essential for optimal performance of the QIAfilter Maxi Cartridge. Do not agitate the QIAfilter Cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging. If, after the 10 min incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipet tip around the walls of the cartridge to dislodge it.)
 8. Remove the cap from the QIAfilter Cartridge outlet nozzle. Gently insert the plunger into the QIAfilter Maxi Cartridge and filter the cell lysate into a 50ml tube. (Filter until all of the lysate has passed through the QIAfilter Cartridge, but do not apply extreme force. Approximately 25 ml of the lysate are generally recovered after filtration.)
 9. Add 2.5 ml Buffer ER to the filtered lysate, mix by inverting the tube approximately 10 times, and incubate on ice for 30 min. (After the addition of
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Buffer ER the lysate appears turbid, but will become clear again during the incubation on ice.)

10. Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT and allow the column to empty by gravity flow. Allow the QIAGEN-tip to drain completely.
11. Apply the filtered lysate from step 9 to the QiaGEN-tip and allow it to enter the resin by gravity flow.
12. Wash the QIAGEN-tip with 2 x 30 ml Buffer QC. (Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.)
13. Elute DNA with 15 ml Buffer QN. (Collect the eluate in a 30 ml tube. Use of polycarbonate centrifuge tubes for collection is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.)
14. Precipitate DNA by adding 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 15,000 rpm for 30 min at 4 °C. Carefully decant the supernatant. (All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4 °C to prevent overheating of the sample. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing supernatant.)

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15. Wash DNA pellet with 5 ml of room temperature 70 % ethanol and centrifuge at 15,000 rpm for 10 min. Carefully decant the supernatant without disturbing the pellet. (The 70 % ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.)
 16. Air-dry the pellet for 5-10 min, and redissolve the DNA in a suitable volume of endotoxin-free Buffer TE. E.g. pellet of 10 mm diameter dissolved in 200 μ l of TE. (Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve.)
 17. Determination of yield: To determine the yield, DNA concentration was determined by UV/Vis spectrophotometry. Quality of DNA: $A_{260}/A_{280} > 1.5$ is considered good.